Universidade de Lisboa

Faculdade de Farmácia

Use of iPSCs for the treatment of neurodegenerative diseases

Catarina Ferreira Mendes Serra

Master of Science (MSc) in Pharmaceutical Sciences

2019

Universidade de Lisboa
Faculdade de Farmácia

Use of iPSCs for the treatment of neurodegenerative diseases

Catarina Ferreira Mendes Serra

Monografia de Mestrado Integrado em Ciências Farmacêuticas apresentada à Universidade de Lisboa através da Faculdade de Farmácia

Supervisor: Assistant Professor Adelaide Fernandes Borralho

2019

Abstract

Stem cells, due to their multi-differentiation potential, have been in these last years, a promising and growing field of study. Since Takahashi and Yamanaka's breakthrough, investigators were granted with means to generate human induced pluripotent stem cells (iPSCs) from patient cells, providing an unparalleled platform for *in vitro* modelling and development of new therapeutic strategies.

The increase of life expectancy leading to a worldwide aging of the population is becoming an ongoing challenge for societies. Adult-uprising of neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS), are among the most difficult human health conditions to model for drug development, lacking any curable treatment, with the present therapies being more focused on relieving the symptoms. The recent progresses in the field of iPSCs have provided a novel route of treatment for neurodegenerative diseases, with the possibility of developing human iPSCs-patient derived, which have successfully differentiated, *in vitro,* into motor neurons, dopaminergic neurons and oligodendrocytes, among others.

This way, iPSCs can be a source of disease-relevant cells, from 2D (two dimensional) to 3D (three dimensional) organoids, suitable for the recapitulation of disease phenotypes, providing an accurate disease model 'in a dish'. They can be used for toxicity studies, drug screening and even allow for the generation of autologous cells, for cell-replacement therapy. Recently, there has been an emergence of new clinical trials, showing the evolving state that this technology has had since its discovery. Nevertheless, for the continuous success of these experiments it will be critical to uncover the molecular mechanisms underlying the reprogramming events when generating iPSCs, focusing on the safety of this technology.

In this essay it is discussed the fundaments of iPSCs technology and their very promising application in the field of neurodegenerative diseases, questioning its advantages and challenges.

Keywords: Alzheimer's disease, Amyotrophic Lateral Sclerosis, induced pluripotent stem cells (iPSCs), Neurodegenerative diseases, Parkinson's disease.

Resumo

As células estaminais, devido ao seu potencial de multi-diferenciação e capacidade auto-replicativa, têm sido, nos últimos anos, alvo de elevada investigação, sendo um campo de crescente conhecimento e novas descobertas. Takahashi e Yamanaka foram os primeiros a derrubar o paradigma das células indiferenciadas, ao descobrirem que fibroblastos, células diferenciadas da pele, poderiam ser revertidas em células estaminais pluripotentes induzidas (iPSCs), permitindo assim a geração de células indiferenciadas a partir de células do próprio doente. Esta notável descoberta permitiu a criação de uma plataforma alternativa para a construção de novos modelos de estudo de doenças, com a possibilidade de testar diferentes estratégias terapêuticas.

Com o aumento da esperança média de vida, a população mundial tem vindo a tornar-se mais envelhecida, constituindo um desafio constante para a sociedade. Neste contexto, existe uma crescente incidência de doenças neurodegenerativas, tais como o Alzheimer, o Parkinson e a Esclerose Lateral Amiotrófica, que constituem algumas das mais difíceis doenças de estudar e para as quais é necessário desenvolver novos fármacos. Estas são, atualmente, doenças sem cura, cuja terapêutica é maioritariamente sintomática e pouco dirigida. Desta forma, o progresso científico das iPSCs possibilitou o surgimento de um rumo inovador para a terapêutica destas doenças, proporcionando o desenvolvimento de iPSCs humanas, derivadas de células dos próprios doentes, com capacidade de serem diferenciadas em neurónios motores, neurónios dopaminérgicos, oligodendrócitos, entre outros.

As iPSCs constituem, desta forma, uma fonte de células relevantes de doenças, na forma 2D (bidimensional) ou 3D (tridimensional), os chamados organoides, sendo pertinentes para o desenvolvimento do fenótipo característico das patologias, capazes de gerar um modelo de estudo ‘em placa’ fidedigno. Estas células podem ser usadas para estudos de toxicidade, triagem de novos fármacos e ainda, gerar células autólogas importantes para terapêuticas regenerativas. É deste modo esperado que as iPSCs possam ultrapassar o problema da rejeição, ao serem usadas células do próprio doente, possuindo grande potencial como terapêutica de transplantação e correção genética, permitindo produzir células saudáveis passíveis de serem transplantadas.

Recentemente, o número de ensaios clínicos com recurso às iPSCs tem vindo a aumentar, comprovando a evolução constante que esta tecnologia tem apresentado, desde o momento da sua descoberta. Todavia, para o seu contínuo sucesso, é

imprescindível que sejam desvendados os mecanismos moleculares subjacentes à fase de reprogramação aquando da geração das iPSCs, procurando atingir a segurança absoluta desta tecnologia científica.

Nesta dissertação serão discutidos os fundamentos das iPSCs e a sua promissora aplicação na área das doenças neurodegenerativas, abordando as vantagens e desafios da sua utilização.

Palavras-chave: Alzheimer, células estaminais pluripotentes induzidas (iPSCs), doenças neurodegenerativas, Esclerose Lateral Amiotrófica (ELA), Parkinson.

Acknowledgments

Finishing this paper represents the culminating of the very hard-working journey that was studying in FFUL and attending the MSc in Pharmaceutical Sciences. I could not have gone through this experience, without the support of some special people that contributed so importantly for this achievement.

Firstly, I would like to thank Professor Adelaide Borralho for giving me the grounds and direction I needed to develop the present report, being available to address my questions and challenges, guiding me through them.

To my family, especially my parents, the ones that made everything possible and always made me believe I could do anything I set my mind for, I say thank you. For all the support, whether it came from a homecooked meal, a rightful phone call, a warm hug, or the advice that I needed to hear, I will always be grateful. To my brother, Alexandra and my three nephews, thank you for all the Sunday laughter. To my grandmothers, thank you for all your worries and kind gestures. I also have to mention my favourite fur ball and family member Kira, the one that never left my side in my toughest writing times.

I am thankful for the great friends that I have in my life. To the friendships that started before I could read and have been with me ever since, I could not be luckier. So, thank you Carolina, Miguel, Ana Lúcia. To my *Coimbrinha* friendships, that are so important in my life, thank you Alma, Inês, Andreia, Joana, Catarina and Sofia. Another special appreciation goes to the ones that always had the power to make me feel at home, Juliana, André, Queijas, Diana, Inês, Luís, Marco, thank you for your constant presence. To Rute, I am lucky to have such a good housemate who was one of the few with enough courage to read this report. To the friends that are more far away but never forgotten, I am grateful for you all.

Finally, I would like to thank my university friends and colleagues for the companionship of these last years, particularly the ones from the very beginning, my three wannabees, Catarina Caçote, Catarina Melício and Sofia Perdigão. They made these last years a lot more fun.

To all of you I say thank you. I hope we can celebrate and share more victories together.

Abbreviations

2D	Two dimensional
3D	Three dimensional
AAT	Alpha-1 anti-trypsin
Aβ	Amyloid-beta
APP	Amyloid precursor protein
AD	Alzheimer's disease
ADME	Absorption, Distribution, Metabolism, Excretion
ALS	Amyotrophic lateral sclerosis
BACE-1	Beta-site amyloid precursor protein cleaving enzyme 1
CiRA	Center for iPS Cell Research and Application
CDK	Cyclin-dependent kinase
CRISPR	Clustered regularly interspaced short palindromic repeats
CSF	Cerebrospinal fluid
DA	Dopaminergic
DALYs	Disability-adjusted life years
EBC	European Brain Council
EMA	European Medicines Agency
ER	Endoplasmic Reticulum
ESC	Embryonic stem cells
EU	European Union
EURALS	European ALS Epidemiology Consortium
eTau	Extracellular Tau
fAD	Familial AD
FDA	Food and Drug Administration
GBA	Glucocerebrosidase
GBD	Global Burden of Disease

IL-17	Interleukin 17
iPSCs	Induced pluripotent stem cells
IVF	*In vitro* fertilization
JNPD	Joint Programme Neurodegenerative Disease Research
JNK	c-Jun N-terminal kinase
L-DOPA	Levodopa
LRRK2	Leucine-rich repeat kinase 2
miRNAs	MicroRNAs
NEP2	Neprilysin-2
NF-kB	Factor nuclear kappa B
NIH	National Institutes of Health (US)
NMDA	N-methyl-D-aspartate
NO	Nitric Oxide
PARK2	Parkin 2/Parkinson protein 2
PAR	Population-attributable risk
PD	Parkinson's disease
PINK1	PTEN-induced kinase 1
pTau	Phosphorylated Tau
PSEN1	Presenilin 1
PSEN2	Presenilin 2
RPE	Retinal pigment epithelium
sAD	Sporadic Alzheimer's disease
SNCA	Alpha-synuclein
SOD1	Superoxide dismutase 1
tTAU	Total Tau
TDP43	TAR DNA-binding protein 43
USA	United States of America

VAPB	Vamp-associated protein B/C
WAS	Wiskott-Aldrich syndrome
ZFN	Zinc Finger Nucleases

Table of Contents

Index of figures

Index of tables

1. Introduction

Stem cells, due to their multi-differentiation potential, have been in these last years, a promising and growing field of study. The recent advances have supported investigators with means to generate human induced pluripotent stem cells (iPSCs) from patient cells, being able, in this way, to overcome the ethical problems associated with embryonic stem cells (ESCs) but also, considering they are patient-matched, reduce the probability of immune rejection after transplantation (1–4).

It was the prodigious contribution of Takahashi and Yamanaka that made it possible to understand how cell fates can be rewound and altered by the ectopic co-expression of specific transcription factors (3,5). The purpose of these scientists was to obtain a different source of pluripotent stem cells, making sure that it would have the same variety of applications as the ESCs but with new and greater prospects for clinical use (4,5) .

Since Takahashi and Yamanaka's breakthrough, iPSCs technologies are emerging as a promising strategy to fill the knowledge gaps between genetic association studies and underlying molecular mechanisms, representing an important advance in the study of diseases, providing an unparalleled platform for *in vitro* modelling and development of new therapeutic strategies (1,6).

IPSC-technology has allowed *in vitro* disease modelling of many diseases and this is especially helpful to investigate pathologies where many different cell types are affected. There has been extensive progress with iPSC-based study and experimentation in the field of neurological diseases, liver diseases, cardiac, haematological, among others. In principle, human iPSCs are able to differentiate into any kind of cell of the human body, hence, patient iPSCs can grant a source of cells that consist of an accurate collection of genetic variants, that correlates with the precise setting of the pathogenesis (6).

Adult-uprising of neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS), are among the most difficult human health conditions to model for drug development (6). Consequently, there is a need to find new and better ways to study these pathologies and iPSCs can most likely be the answer.

In this essay the fundaments of iPSCs technology and their very promising application in the field of Neurodegenerative diseases are discussed: (i) What is known about these diseases and how can iPSCs help establish new models of study? (ii)

What are the advantages of this technology comparing to the existing ones? (iii) How can iPSCs be used as therapeutic means in neurodegenerative disorders? (iv) Are there any challenges to overthrow? These are some questions that will be reviewed and debated along the essay.

2. Methods

The research for the present essay was entirely electronic. Initially it consisted on a global research of the topic, using different platforms and databases like PubMed, Scielo, Nature, and websites from organizations such as: WHO (World Health Organization), EBC (European Brain Council), NIH (National Institutes of Health), EMA (European Medicines Agency) and JNPD (EU Joint Programme Neurodegenerative Disease Research), to review the most updated published literature. It was also useful to search *clinicaltrials.org* database to understand what impact iPSCs have in clinical trials of neurodegenerative diseases.

The first part of the investigation was obtained with a simple research with the key-words: “iPSCs”; “neurodegenerative disease”; “induced pluripotent stem cells”. As the investigation for this essay was evolving some other key-words were used, such as: “Alzheimer”, “Parkinson”, Amyotrophic Lateral Sclerosis”, “ALS”, “regenerative medicine”, “cell therapy”, “3D organoids”, “cell dish”, “transplantation”, “drug screening” and “models of study”.

There was a concern to use recent and current studies, selecting articles and reviews from the latest years. Moreover, the papers and data chosen for this research were carefully evaluated in order to use trustworthy and reliable information.

3. Neurodegenerative diseases

The increase of life expectancy leading to a worldwide aging of the population is becoming an ongoing challenge for societies. The rising growth of health problems is one of the main concerns and more specifically the escalating number of people suffering from neurodegenerative diseases, which typically develop mid- to late life. (7,8)

Neurodegenerative diseases are often described as pathological conditions in which the progressive atrophy of neurons and tissue occurs, culminating in apoptosis and severe degeneration of the affected patient brain regions. It corresponds to a loss of neuronal function and leads to impaired movement and cognition that will vary according to the type of neurodegenerative disease. (6,8,9) Each disease has its specific pathological mechanism affecting a different kind of neuron, with some causing more cognitive and memory lost and others causing impairments in movement, speech and the ability to breathe (6,8,10).

Vast research has made it possible to develop and improve the knowledge about the pathophysiology of neurodegenerative diseases. There is much heterogeneity, but also many overlapping features with a common molecular mechanism that anticipates the neuronal death, such as mitochondrial dysfunction, axonal damage and anomalous protein aggregation (6,8,11). All through the numerous neurodegenerative diseases, there is a typical factor: the appearance and spreading of these aberrant protein aggregates throughout the brain. In the last decade, the evidence has become more clear but still with much to discover, documenting the hallmarks of each disease, including the α-synuclein and ubiquitin Lewy bodies in PD and Amyloid β (Aβ) plaques and phosphorylated Tau (pTau) - containing tangles in AD (6,11,12).

Currently, there is no cure for any of these diseases, with the present therapies being more focused on relieving the symptoms. Accuracy on the diagnose is crucial, as it allows a better guidance to the right treatment, management and an improved prognostic (8).

In the most recent years, it has become clear that not only the disorders of the brain have become more prevalent, but are also, presently, adding to a greater disease burden. Overall, this is mainly due to disability, considering the impact it has on the daily capacity of patients, rather than ending in premature death as it happens in diseases like cancer or cardiovascular conditions (13–15).

According to data from WHO 2008, “neurological disorders included in the neuropsychiatric category contribute to 2% of the global burden of disease, in 2005” (15). To assess the global burden of disease, the study by WHO measures the disability-adjusted life years (DALYs), that provides a measure of the future healthy life lost (years expected to be lived in full health), as a result of the incidence of specific diseases and injuries. The DALYs are obtained as a sum of two other components: premature mortality (years of life lost because of premature mortality or YLL) and disability (years of healthy life lost as a result of disability or YLD, weighted by the severity of the disability).

In this same study by WHO, there is a projection concerning the contribution of neurological disorders in DALYs for 2005, 2015 and 2030. Neurological disorders contributed to 92 million DALYs in 2005 and is projected to increase to 103 million in 2030 (approximately a 12% increase). More specifically, AD and other dementias are projected to show a 66% increase from 2005 to 2030 (15).

Table 1 - Number of DALYs for neurological disorders and as percentage of global DALYs projected for 2005, 2015 and 2030. From: (15)

Cause category	2005		2015		2030	
	No. of DALYs (000)	Percentage of total DALYs	No. of DALYs (000)	Percentage of total DALYs	No. of DALYs (000)	Percentage of total DALYs
Epilepsy	7308	0.50	7419	0.50	7442	0.49
Alzheimer and other dementias	11078	0.75	13540	0.91	18394	1.20
Parkinson's disease	1617	0.11	1762	0.12	2015	0.13
Multiple sclerosis	1510	0.10	1586	0.11	1648	0.11
Migraine	7660	0.52	7736	0.52	7596	0.50
Cerebrovascular disease	50785	3.46	53815	3.63	60864	3.99
Poliomyelitis	115	0.01	47	0.00	13	0.00
Tetanus	6423	0.44	4871	0.33	3174	0.21
Meningitis	5337	0.36	3528	0.24	2039	0.13
Japanese encephalitis	561	0.04	304	0.02	150	0.01
Total	**92392**	**6.29**	**94608**	**6.39**	**103335**	**6.77**

Globally neurological disorders constitute 16.8% of the total of deaths, with AD and other dementias being estimated to represent 2.84% of this total, in high income countries, in 2005 (15).

In the EU (European Union), mental disorders are accountable for a tremendous part of the overall burden of disease: nearly 1 in 3 of all years of life lost due to premature death in women, and nearly 1 in 4 in men, are a result of a brain disorder (13).

In a report from 2011, looking at the 27 European countries, it was estimated that 164.7 million had a brain disorder, meaning that almost 38% of the EU suffered from

some type of brain disease. It predicted that the combination of all neurological disorders results in 30.1% of the total disease burden in females and 23.4% in males (13).

The EBC, a federation of European wide organizations with an interest in the brain and its disorders, estimated that, in 2010, the total costs of disorders of the brain was 798 billion €. This total comprises 19 groups of disorders (addictive disorders, affective disorders, anxiety disorders, brain tumour, childhood and adolescent disorders - developmental disorders, dementia, eating disorders, epilepsy, mental retardation, migraine, multiple sclerosis, neuromuscular disorders, PD, personality disorders, psychotic disorders, sleep disorders, somatoform disorders, stroke, and traumatic brain injury) studied in all 27 EU member states plus Norway, Iceland and Switzerland (14). The most part of the predicted costs of brain disorders was direct costs, 60%, divided into direct healthcare costs (295 billion, 37%) and direct non-medical costs (186 billion, 23%), whereas the remaining 40% were indirect costs (315 billion) (14,16).

The cost per person with a disorder of the brain is highly variable by disorder. On average, it ranged between 285 million € for headache and 30 000 million € for neuromuscular disorders. The estimated total costs for dementia were 105.163 million € and for PD were 13.933 million €.

Dementia was estimated to contribute with much higher direct non-medical costs, representing 84% of the total (88.214 million €), when compared with direct medical costs (16.949 million €), and was considered has having non-existent indirect healthcare costs. As for PD, the direct medical costs were higher (7029 million €) than the non-medical (5519 million €), and the indirect costs (1386 million €) (14).

The mean costs per capita, in Europe, was estimated at 1550 million €. Portugal had a predicted total expense of 13.130 million €. In the specific context of dementia and PD, Portugal had total costs of 1135 and 172 million €, respectively (14).

Neurodegenerative diseases ought to be considered Europe's topmost health challenge of the 21st century. Currently there is no absolute cure, the treatments most often are not adequate, nor specific to the type of disorder, furthermore there is a lack of preventive measures. All of these constitute concerns and problems that need to be addressed and solved, there is an immediate need for action. Nevertheless, along with the need for new research and pharmaceutical development, it is crucial to make a transformation, to rethink the strategy and standards for mental healthcare and work towards reducing the stigma and unawareness that still exists nowadays (13,16).

3.1 Alzheimer's disease

AD is a progressive neurodegenerative disease, considered the most common form of dementia, possibly contributing to 60–70% of all cases. (*World Health Organization, 2015)* Although it can occur in people of young age, it is typically a disease of the elderly, with prevalence rates rising substantially between 65 to 80 years old, even if it is not a normal part of ageing (17).

AD is clinically defined by a progressive decline in learning and memory capacities, with acute cognitive disfunction during the late stage of the disease. It affects wide areas of the brain, causing brain volume reductions and hippocampal degeneration. Initially, abnormalities are commonly present in the brain tissue involving the temporal and frontal lobes, gradually progressing to other areas of the cortex (8,18,19). The typical pathological hallmarks are the accumulation of insoluble forms of Aβ (amyloid beta) peptides in plaques and formation of neurofibrillary tangles containing protein Tau along with reactive microgliosis, dystrophic neurites, and loss of neurons and synapses (18,19).

Currently, Aβ, APOE and Tau are three elements with significant evidences as triggers of AD. Aβ is the main constituent of plaques and is originated from APP (amyloid precursor protein) through a sequential cleavage by catalytic subunits of the γ-secretase complex. Most research suggest that Aβ accumulation is based on the alteration of peptide conformation leading to forms a high β-sheet structure, which is critical in AD pathogenesis (20). One of the first reports linking APOE to AD pathology was its immunoreactivity in Aβ deposits and neurofibrillary tangles, hallmarks of AD pathology. APOE is a protein with three common isoforms in humans, with high expression in the brain, and it is involved in the normal catabolism of triglyceride-rich lipoproteins, being considered the strongest genetic risk factor for developing of AD (21). Numerous studies have shown that total tau (t-tau) and phosphorylated tau (p-Tau) levels are increased in both the brain and cerebrospinal fluid (CSF) of patients with AD, even though the mechanisms for such occurrence are not known (22).

The etiology of AD is still uncertain, with both genetic and environmental factors likely to be involved. Mostly, genetic forms of AD culminate in disease onset before 60, being appointed as familial AD. In opposition, the idiopathic AD is more common, and has a higher incidence after 65, being termed the sporadic or late-onset AD (23). The familial AD is associated with gene mutations all involved in Aβ plaques formation, including amyloid-β precursor protein APP, presenilin1 (PSEN1) and presenilin2 (PSEN2) , the γ-secretase complex (19,23). Nevertheless, the causes of sporadic AD

are more difficult to define. A few genetic factors have been reported, with the e4 variant of the gene APOE, APOE4 allele, being estimated to contribute to around 50% of the cases. ApoE4 is a carrier of Aβ protein and could therefore be related to the accumulation of amyloid plaques. In addition, the deficient ApoE4 binding to tau protein could lead to fewer phosphorylation of the protein, resulting in aggregation into neurofibrillary tangles (8,24).

According to the *World Alzheimer Report* of 2015, from *Alzheimer's Disease International*, the number of people living with dementia worldwide in 2015 was estimated at 47.47 million, reaching 75.63 million in 2030 and 135.46 million in 2050. In the whole world, there are closely 10 million new cases of dementia every year, implying there is a new case every 3.2 seconds, with population ageing being the main driver of this projected increases (25,26). It was estimated that Est Asia is the region in the world with the highest amount of people living with dementia, 9.8 million, followed by Western Europe, 7.4 million (25).

Accurate measurement of AD prevalence rates is a difficult challenge, with two main factors contributing: there is no definitive diagnostic test and most of its signs and symptoms are shared by several other forms of dementia (17,27). Nevertheless, there are projections estimating that by 2050 AD will have a worldwide prevalence of 106.8 million, with 1 in 85 persons living with AD (28).

There are only few complete trials about AD correlation with risk factors. A 2010 report from the State-of-the-Science Conference convened by *US National Institutes of Health* (NIH), declares that "firm conclusions cannot be drawn about the association of any modifiable risk factor with cognitive decline or Alzheimer's disease" (29). On the other hand, there is compelling evidence from studies defending the benefits of reducing some risk factors such as: improvement of cardiovascular health, having better physical activity, control tobacco consumption and having higher levels of education (17,26,30).

Studies suggest that, aside from age, which is the strongest risk factor for dementia, cerebrovascular disease is the most reported correlated event (17,26,30). A study from 2014, estimated the population-attributable risk (PAR) of AD worldwide regarding seven modifiable risk factors that have consistent evidence of an association with the disease (diabetes, midlife hypertension, midlife obesity, physical inactivity, depression, smoking and low educational attainment) (30). The results concluded that, the largest amount of AD cases in the USA and Europe might be related to physical inactivity, with low educational attainment being correlated to around one in five cases

of AD worldwide. This study infers that approximately one third of the AD cases could be related to the seven potentially modifiable risk factors considered, and by decreasing them it would be possible to reduce worldwide prevalence of AD between 8% and 15%, by 2050 (30).

This way, primary prevention of AD should be focused on improving the access to education and reducing risk factors of cardiovascular disease such as hypertension, diabetes, tobacco consumption, physical inactivity and obesity (25,26).

Dementia shortens the life of those affected. Nonetheless, in opposition with other conditions, its impact comes from the chronic disability and need for care, rather than years lost from premature mortality. It is on the top 10 diseases with higher burden among older people (25).

None of the pharmacological treatments available nowadays for AD has the capacity to slow or completely cease the deterioration and loss of neurons, which causes the symptomatology and make it a fatal disease. There are five medicinal products approved by the U.S. Food and Drug Administration (FDA) and four approved by the European Medicines Agency (EMA) available in Portugal that can temporarily diminish symptoms and help manage the disease. Those medicines are cholinesterase inhibitors including: donepezil, rivastigmine and galantamine, which enhance cholinergic transmission in relevant sections of the brain; and a N-methyl-D-aspartate (NMDA) receptor antagonist, memantine, used on a moderate to severe AD, that regulates the activity of glutamate, improving behavioural symptoms. FDA also approved a combination of donepezil and memantine for the moderate to severe form of AD. Plenty of failures in AD drug development have occurred, with both small molecules and immunotherapies failing to achieve success. The AD drug development pipeline is relatively small, and the rate of success of AD clinical trials is limited, with most of the recent trials addressing medicines intended to improve cognition and a therapy more directed to earlier stages of the disease (31,32).

As voiced by Dr Margaret Chan, Director-General of the *World Health Organization*, at the "*Global Action Against Dementia"* conference that happened in Geneva, in 2015 "*I can think of no other disease that has such a profound effect on loss of function, loss of independence, and the need for care. I can think of no other disease so deeply dreaded by anyone who wants to age gracefully and with dignity. I can think of no other disease that places such a heavy burden on families, communities, and societies. I can think of no other disease where innovation, including breakthrough discoveries to develop a cure, is so badly needed*."

3.2 Amyotrophic Lateral Sclerosis disease

ALS is a heterogeneous neurodegenerative disease that is characterised by the degeneration and death of upper motor neurons (neurons that project from the cortex to the brainstem and the spinal cord) and lower motor neurons (neurons that project from the brainstem or spinal cord to muscle) leading to symptoms like muscle atrophy, paralysis and finally death induced by failure of the respiratory muscles (33–35). Being a complex disorder ALS affects different cell-types, including astrocytes, microglia and oligodendrocytes, and they all seem to contribute to the death of neurons (36).

The initial symptoms of the disease vary a lot between patients: some of the patients can present the spinal-onset disease, with muscle weakness of the limbs, but others can display a bulbar-onset disease, defined by dysarthria and dysphagia (33). Actually, there are no early methods to diagnose ALS and life expectancy is usually 3-5 years after the first symptoms occur (35).

According to the European ALS Epidemiology Consortium (EURALS) and a research by Logroscino *et al*., in Europe, the incidence ranges from 2 to 3 cases per 100,000 individuals, as ALS is considered to be a rare disease (37).

In terms of the causes of the disease, in most cases, they are unknown. However, there are some risk factors, with the most important ones being the influence of heavy metals and toxins, smoking, severe brain injuries, increased motor activity, latent viral or non-viral infections, and autoimmune reactions (35). Even though, the majority of ALS cases are sporadic, there are some patients, 5 to 10%, that have the familial form of ALS, caused by a dominant autosomal mutation and of those, four genes account for up to 70% of all cases, namely, C9orf72, TARDBP (encoding TAR DNA-binding protein 43, TDP43), SOD1 (encoding superoxide dismutase) and FUS (encoding RNA binding protein FUS) (38).

There are no definite cures for ALS. Even though more than 50 drugs with different mechanisms of action have been investigated for the treatment of ALS, there were only two, edaravone and riluzole, that have achieved approval success. Riluzole was the first FDA-approved treatment for ALS and has a mechanism of action that is poorly understood. In its first trial, riluzole increased survival by 3 months, after 18 months of treatment, compared with placebo, but does not alleviate the symptoms (39). More recently, the antioxidant edaravone, proved to be able to slow the rate of ALS progression in highly selected patients with an early onset fast progressing disease. Despite the being approved by FDA, edaravone is not yet approved by the European

Medicines Agency (EMA) and there was actually a withdraw of the marketing authorisation application in the present year, without any impact in the current clinical trials (33).

Some progress has been made concerning ALS biomarkers, but not as quite when comparing to other brain disorders. However, the last decades have brought some important knowledge about the disease pathologic heterogeneity, there have been some extensive efforts to find new effective medicines and an extensive pipeline of new therapeutics for ALS is available (40). For example, symptomatic therapies, such as tirasemtiv, based on correcting respiratory function in patients with ALS, which is currently in phase III trials. The future is focusing on targeting therapies, for specific subgroups of patients and biomarkers that are personalized to the persons' disease subtype (33).

3.3 Parkinson's disease

PD is the second most prevalent neurodegenerative disorder after AD and represents the most common movement disorder.(41) In 2016, Global Burden of Disease, Injuries, and Risk Factors Study (GBD) examined the epidemiology of Parkinson's in different parts of the world. Among other neurological diseases, PD was the fastest growing in prevalence, disability, and number of deaths (42). As the population ages and life expectancy increases, it is projected that the number of individuals doubles again in the next generation, like it did between 1990 and 2016 (42).

The incidence of PD can be associated with risk and protective factors, with age being the most important risk factor, common to all neurodegenerative diseases. Some other risk factors seem to be the exposure to any-type of pesticides, herbicides, and solvents, showing significant association between this disease and workers from specific industry sectors (agriculture, metallurgy, textiles) (43,44). Interestingly, cigarette smoking is associated with a lower risk of developing PD (45). Despite such results, it is important to perform greater number and more precise studies, to prevent information bias, or residual confounding, as it happens in some environmental risk factors researches (46).

According to the GBD epidemiological study, in 2016 "*6.1 million (95% UI 5.0–7.3) individuals worldwide had Parkinson's disease, of whom 2.9 million (47.5%) were women and 3.2 million (52.5%) were men*" (42). Also, the number of deaths was 2.6 times higher and the number of DALYs was 2.5 times higher in 2016 than in 1990. This

same study corroborates that PD is about 1.4 times more frequent in men than women and environmental factors to which men are more frequently exposed might contribute to such pattern (42).

The physiopathology of PD is characterised by neuronal death, affecting mostly the dopaminergic (DA) neurons of the *substantia nigra pars compacta,* in the central nervous system. As DA (dopaminergic) neurons have an important role in controlling motor functions, the disruption of the basal ganglia's motor control network will result in the most known and noticeable symptoms of PD: bradykinesia, resting tremor, rigidity, and postural instability (47,48). Nonetheless, PD is a systemic disease and as it progresses, other regions of the brain are affected, causing other type of symptomatology, such as anxiety and mood changes (49).

Histological hallmarks of PD are the presence of α-synuclein (small protein encoded by the α-synuclein gene, designated SNCA for synuclein alpha) containing Lewy bodies and Lewy neurites in the *substantia nigra*, with this α-synuclein accumulation becoming more widespread in the brain with the progression the disease (47,49).

The etiology of PD is not defined in most of the identified cases, however the majority of those are sporadic and idiopathic, resulting from a combination of environmental factors and genetic background (48). Despite that, almost 5% of the cases are considered to be familial and triggered by known gene mutations, with some of these genes having had great amounts of researched: LRRK2 (leucine-rich repeat kinase), SNCA, PINK1(PTEN induced putative kinase 1), and PARK2 (parkin) (50).

Several mutations in LRRK2, the most frequent cause of familial PD, have been identified, with G2019S mutation in the kinase domain of LRRK2 being the most common, in terms of familial PD (50). Multiplications in the SNCA gene, have been linked, in numerous studies, to familial PD, with a gene triplication leading to an earlier onset and quicker progression of the disease, which illustrates that disease severity is dependent on α-synuclein expression degree (51). Both these genes suffer mutations that will cause autosomal dominant PD, contrarily to what happens with loss-of-function mutations in PINK1 and PARK2, which cause recessive PD and are associated with an early onset. It seems that, in a small proportion of sporadic PD patients, LRRK2 and SNCA are also mutated (48,50,51).

It is important to fully understand the function of PD-related genes, since, in the idiopathic form of PD there could be similar pathways, which would be relevant at a therapeutic level. Until now, there are a few FDA and EMA-approved therapies, being

the administration of L-DOPA (levodopa) and dopaminergic agonists, the first line approaches. Other medicines such as anticholinergic, MAO-B inhibitors, COMT inhibitors are also approved and deep brain stimulation of the bilateral subthalamic nuclei is available as well as a non- pharmacological approach. The decision of which therapy to follow depends on the state of the disease and the person's characteristics. However, these are palliative treatments which are not a cure for the disease (48). One critical factor is that the variety of systems and neurotransmitters affected in PD pathology lead to highly irregular patient responses to L-DOPA treatment, suggesting that each patient should have a personalized treatment approach (47).

The difficulty for finding treatment for PD resides in the fact that only after a big percentage of DA neurons have died, PD symptoms will manifest.

Novel genes associated with PD are being discovered and with that, new therapeutics are emerging. Despite that, therapeutic developments have been hold up by the absence of optimal *in vitro* and *in vivo* experimental models that can be predictive of human disease (40,48).

4. Models to assess pathogenesis and assay therapeutic interventions

4.1 Challenges in drug discovery, classic cellular and animal models

The major limitation in the study of neurodegenerative diseases is the lack of an *in vitro* model, that can faithfully replicate the complexity and fragility of the human brain. If such model could be constructed and studied in association with animal models new and improved information about these diseases, with good outcomes for the patients, should arise. Despite of the great investments made, to this day, attempts to develop *in vitro* models have been in hindered and have not been so successful (40,52,53).

Neurodegenerative diseases still constitute an absolute challenge for drug development and clinical trials, with no complete cures or successful disease-modifying therapies yet discovered (40,53,54). The pattern is to have a great reliance on studies which use nonhuman genetically engineered models and are able to study specific features of the disease at a cellular or molecular degree. However those models fail to represent other significant phenotypes of human disease and that do not entirely reflect drug efficacy (53). There are quite a few limitations for the study of neurodegeneration, considering the difficult access to tissue and samples of the

disease-affected areas, the incomplete understanding of these diseases molecular mechanisms, the modest knowledge of neurological biomarkers and the lack of a global standardized database consorting all the neurodegenerative diseases (40,53).

Only a limited number of drugs are currently available for the treatment of neurodegenerative diseases. Despite increased investment in research and development in the last decades, that has not translated into a sustained raise in the discovery of new drugs by pharmaceutical companies (55). The classical drug discovery pipeline holds different stages and it is definitely uncertain, with the duration of time for regulatory review and approval of neurological medicines being the longest across all disease areas: 12.6 years in development and regulatory approval compared with, for example, 6.3 years and 7.5 years for cardiovascular or gastrointestinal indications, respectively (40,54,56) .The success rates in central nervous system drugs is considered low but utterly explicable due to the outright complexity of the brain, the existence of a blood-brain barrier that complicates drug penetration and also the high tendency for these type of pharmaceuticals to cause side effects (56).

Most of the studies and drug trials have been based on cellular lines grown in the laboratory and a large variety of experimental animal models (mouse, fruit fly, dog, nematode worm, and even baker's yeast), with rodents being the most used.

Animal models represent a valuable resource for experimentation, phenotypic and preclinical testing, making an important platform for translational studies, which are not possible in human patients (57,58). The transgenic animal models enable the study of the pathogenic mechanism of the diseases and predict drug efficacy for symptomatic treatment (59–61). Different type of evaluations are made in these models, such as the assessment of cognitive abilities, the examination of cerebral vascular anomalies and the behavioural/motor tests' correlation with neuromuscular degeneration (57).

Although discoveries from animal models may have brought innovation, especially on predicting drug efficacy for symptomatic treatment, they have also failed many times in replicating disease phenotypes and have not been so helpful for the identification of medicines that can act as disease modifiers. That may be due to the microenvironment, considering most neurodegenerative diseases have a sporadic etiology deriving from complex interactions of genetic and environmental risk factors and also the specie-specific differences and short lifespan of most animals used (52,53,57).

In terms of *in vitro* models, different types have been used in neurodegenerative disease's studies: cell lines derived from populations of cells from a multicellular organism (that can be either naturally or artificially immortalized), primary cultures and three-dimensional cultures (organotypic culture). Immortalized fibroblasts, tumours from the nervous system, or immortalized neuronal progenitor cell lines for *in vitro* assays are still the most used (53). By using this cell lines approach it is possible to cover a large range of drugs and select the most promising for *in vivo* studies, which have led to the success of a vast number of neuroprotective compounds. This model has major advantages such as its high reproducibility and indefinite proliferation, but also considerable downsides. Immortalized cells are unable to reproduce the morphology and physiology of a neuronal cell, they do not express important levels of synaptic proteins when compared to neurons and retain a high proliferative state that represents a significant contrast to neurons, which do not undergo division (62–64).

Hence, these models often fail to truthfully reproduce disease mechanisms and to overcome such obstacles, it would be significant to access patient-derived cells, so that disease relevant neural cell-types could be obtained (neurons specific to each disease). Such access is challenging as biopsies or resections are rarely or never performed in patients with these diseases and even if *post-mortem* samples from the nervous system can be obtained, those will most likely be damaged by terminal manifestations of the disease (52,53).

Knowledge breakthroughs have been made in the areas of stem cell and biologic reprogramming. The recent progresses in the ability to reprogram patient somatic cells into iPSCs have provided a novel route to develop disease-relevant patient-specific cells for *in vitro* modelling, generating human models of human disease (65,66).

4.2 What are iPSCs

Human pluripotent stem cells are characterized by the capacity to infinitely renew in culture, while keeping the ability to differentiate into any cell type, including neurons, when given the appropriate conditions (52,67).

The concept of cell pluripotency goes back to 1891, when Driesch found that when he separated the two cells of the early sea urchin blastocyst he observed the development of two complete urchins (68). It was much later that related advances were made, with studies of the embryo and blastocysts, strengthening the theory that

the cells from the inner mass of the mouse blastocyst were pluripotent (69,70). Pluripotency is the highly specialized ability of the inner cell mass (IMC) of the blastocyst to differentiate into any other cells of the human body, except the placenta (71). In 1961, Till and McCulloch had also an important contribute, revealing the self-renewal capacity by any living cells (72).

Later studies defined cells according to their differentiation and self-renewal potential, being denominated stem cells. Depending on their origin and differentiation, stem cells can be Adult Stem Cells or Embryonic Stem Cells (ESC), being classified as unipotent, multipotent, oligopotent, pluripotent and totipotent (73). ESC are derived from totipotent cells of the early mammalian embryo and are capable of unlimited, undifferentiated proliferation *in vitro*. The era of culturing pluripotent stem cells in a dish began with the first successful human embryonic stem cells isolation happening in 1998, by Thomson and colleagues. They produced cleavage stage human embryos, by *in vitro* fertilization (IVF), for clinical purposes, which were donated by individuals after informed consent and institutional review board approval (74). With the promising advances in the study of human development there were numerous new possibilities, including the chance to model diseases, discovery pathologies mechanisms and to use cell therapy to treat incurable diseases.

However, the ethical problems regarding the use of human embryos led to a lot of controversy and studies were restricted. Most ES cells represented generic cells isolated from presumably normal embryos and were not matched to a particular patient, so their use for transplantation would lead to rejection (75). Thus, even though human ESCs, have been strongly expected to be a key to the treatment of incurable diseases, such as PD and spinal cord injuries, its acceptance has faced major struggles, including ethical concerns regarding the use of human embryos and immune rejection after transplantation (76).

It was in 2006 that a landmark in cell biology was reached, when Takahashi and Yamanaka introduced the concept of iPSCs, by developing stem cells with ESCs related properties (5). They described the reprogramming of human somatic (skin) cells to pluripotency through the expression of four transcription factors: OCT4, SOX2, KLF4 and c-MYC. This first generation of iPSCs were similar to ESC in proliferation, morphology, cell marker gene expression and ability to originate teratomas. That discovery won Yamanaka the Nobel Prize for Medicine in 2012. It offered an unprecedented opportunity to overcome obstacles of other study models and had a distinctive potential to be used in made-to-order therapies with autologous cells.

Human iPSCs and ES cells can be considered very identical biologically, but their origin is different, as iPSCs are pluripotent cells that are derived artificially, by ‘labored’ expression of endogenous pluripotency genes induced by exogenous reprogramming transcription factors, while ESC are pluripotent cells that derive from preimplantation stage embryos. These two cell types are collectively described as human pluripotent stem cells have both been used for modelling human genetic diseases, with the earlier models using ESCs. As iPSCs technology emerged, it became a favoured choice, due to its easier availability and absence of ethical problems which affect human ESC (65).

4.3 How are iPSCs generated

Since Yamanaka’s big discovery in 2006 (5), several methods with different reprogramming factors combinations, have been described, with improvement in their efficiency.

In theory, iPSCs can be generated by using any kind of somatic cell, if the right reprogramming factors are used and the most convenient method is chosen for its insertion (77). The procedure steps can be summarized in: initial establishment of the cell culture; secondly, the phase of induction into iPSCs, with the correct choice of reprogramming factors, and lastly, the characterization (morphological and physicochemical methods) and expansion of the iPSCs.

Hence, to generate iPSCs from somatic cells, introduction of reprogramming factors is needed and that can be either achieved with integrating methods or with a non-integrating system.

Integrating methods result in human iPSC lines that carry randomly distributed insertion of transgenes and the vector gets integrated in the cell genome. Non-integrating approaches lead to human iPSCs without any resultant permanent genetic modification, including viral and non-viral delivery, including transient transfection with episomal vectors, recombinant proteins, Sendai virus, adeno viral transduction, synthetic mRNAs or mature double-stranded microRNAs (miRNAs). The use of nonviral methods or non-integrating viruses could avoid genomic insertions, in this way reducing associated risks when human iPSCs are used for clinical applications. Although different combinations have been tried with various rates of success, the general view is that with fewer reprogramming factors used, the safer resulting iPSCs will be. Yet, it might be harder to achieve complete reprogramming with a smaller number of factors. Overall, even though it is not easy to choose the ideal method, it is

crucial to define precise approaches to evaluate each iPSC clone and carefully select subclones, before using them for clinical purposes. As soon as the reprogramming factors are delivered to the somatic cells, they will promote its transformation from somatic cell into iPSCs (5,76,78–80).

In addition to fibroblasts, easily accessible through a skin biopsy and were the first successful somatic cells used (81), a variety of other cell types have been tried out such as hepatocytes (82) peripheral blood cells (83), blood progenitor cells (84), keratinocytes (85), circulating T cells (86), kidney mesangial cells (87) and even human urine cells (88). To obtain somatic cells from donors through a simple and safe procedure is the most important factor, and that can be achieved with simple biopsies or when the patient undergoes surgery.

A notable amount of protocols describes differentiation to neurons, smooth muscle cells, cardiomyocytes, hepatocytes, and haematopoietic cells. The result is generally a heterogeneous population of relatively immature differentiated cells that will further require a subtype selection, usually based on their morphology (89).

Human iPSCs have had an immediate appeal for the establishment of human ‘disease in a dish’ models and have been selected for medicines screening (drug discovery and toxicity studies) and clinical studies including regenerative medicine.

4.4 iPSCs as disease models: cell cultures and organoids

Disease modelling using primary patient-derived cells is helpful for studying human diseases etiology and to develop therapeutic strategies for such.

iPSCs can be a source of disease-relevant cells, that otherwise would be inaccessible, for example neurons and cardiomyocytes. They can easily model human disease, due to their intrinsic properties of self-renewal and potential to differentiate into nearly any cell type, especially diseases with defined genetic causes (90). Numerous studies have confirmed that human iPSCs can be used to model genetic diseases, by showing that descendant cells affected by the disease in patients can recapitulate disease characteristics *in vitro* (91).

Moreover, considering iPSCs can be developed from the patient relevant cells, they offer a chance for personalized disease modelling and the promotion of customized therapies.

This way, disease modelling using human iPSCs begins with the process of deriving iPSCs containing the disease-causing mutation, that are then differentiated

into disease-relevant cell types. The obtained cells are subsequently used to reveal disease etiology and to identify pathological mechanisms.

With the advance of technology, it is possible, nowadays, to introduce specific genetic changes into iPSCs, allowing for the correction of gene mutations that cause diseases and in opposition, introduce mutations in iPSCs derived from patient cells with no disease. These developments led to the establishing of isogenic iPSC lines, in which the disease mutation is corrected, ensuring in this way, the trustworthy recognition of the true pathology and deflecting any possible divergences from genetic background or epiphenomena resulting from line-to-line variations. Such approach assumes particular interest when modelling sporadic or polygenic diseases, where phenotypic differences are anticipated to be minor and are often thought to be induced by mixed small-effect genetic variants combined with environmental factors (for example in a big part of AD cases) (92).

Heart diseases were among the first diseases to be studied with human iPSCs, but various forms of diseases which are caused by some type of deficiency have also been studied with this technology (93). Numerous syndromes which are caused by the presence of one or more additional copies of a chromosome, have also been researched using the iPSCs. For example, Briggs *et al.* resorted to iPSCs to study Down Syndrome and identify the molecular pathways behind its occurrence (94).

In the field of neurobiology, a lot of progresses have been made using iPSC as disease models, specially studying neurodegenerative diseases (for example iPSC-derived neurons have been used to model AD and PD). Another useful application of iPSCs is in the constitution of 3D organoids, a better way to model interactions between different cell types. These have been generated for a variety of organs, including the brain, retina, stomach, lung, intestine, liver, kidney using pluripotent stem cells both from mice and human (95).

The development of 3D in vitro cultures, in which cells organize into complex structures with the name “organoid”, emerged with the need to develop a more accurate model of tissue development, one that two-dimensional cell cultures could not deliver (96). IPSCs have the capacity for self-organization and can develop into 3D structures resembling mini-organs, including the brain. With the combination of the organoid method and the recently developed gene editing technologies (such as transcription activator CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats), researchers have now the ability to study evolving processes and study diseases at an unparalleled degree.

Organoids have been developed for different and numerous applications, due to the fact they enable the study of cell-to-cell interactions in a context that mirrors the

human physiology and cellular microenvironment. This way, they have been used to model human organ development and diseases, to test therapeutic effects of medicines and also be used in cell transplantation (95,97,98).

Human brain organoids represent a new way to investigate neurological disease mechanisms that would otherwise not be possible to study, being particularly interesting for investigations that demand live and operating tissues, such as live imaging of dynamic cell mechanisms and electrophysiological assays (9). Furthermore, increasing evidence implies that most neurodegenerative diseases are not exclusively diseases of decaying neurons, but are also affected by non-neuronal cells in the brain, such as glial cells, which are actually more prevailing in the brain and the central nervous system than neurons itself, and have significant roles in the disease evolution (99). The 3D brain organoids, this way, should incorporate these neuron-glial interactions to grant a better clarification of cells non-autonomous disease mechanisms.

Even though these brain organoids exhibit plenty advantages when compared to animal models, they also offer some limitations and challenges with a chance for improvements. One of those challenges is related to the absence of a surrounding supportive tissue and body axes, that prevent brain organoids from organizing into the same pattern as *in vivo,* also the absence of vascularization (important to see the interaction of blood vessels and the brain tissue itself) and a tendency for the generation of heterogenous organoids with significant variability. There is, therefore, a need to increase efficiency and reproducibility, when comparing to 2D cultures (96,100,101).

Further upgrading of the organoid protocols coupled with technology development, would benefit a research of more complicated interactions on the brain, such as neuron–glia interactions and neural circuits. The development of a more standardized organoid culture medium, along with a more defined extracellular matrix, would simplify the generation of a highly reproducible organoid system Above all, it would give support to model a larger variety of neurological diseases, including the ones of the developing adult and aging brain.

4.5 iPSC-based drug discovery

Animal models or *in vitro* cellular models are limited by their inability to fully replicate human physiological conditions and phenotypical characteristics. Animal models are not suitable for drug toxicity testing, considering the different responses and levels of toxicity that can change depending on the animal tested. Most

importantly, a new medicine or therapy must always be tested on human cells or test models, meaning it would be convenient to have a method that could directly extrapolate the results to humans (77).

Patient-derived iPSC models make it possible to recapitulate disease phenotypes in a culture dish. For toxicity studies, iPSCs originated from diseased and healthy cells are differentiated into neurons, hepatocytes, among others. These are very important studies and constitute one relevant tool to exclude new therapeutic molecules and prevent them from progressing into clinical trials. The absence of early toxicity detection in human tissues leads to more costly and highly time-consuming drug development processes. For this reason, establishment of toxicity models capable of an accurate prediction of cardiotoxicity, neurotoxicity and hepatotoxicity, before clinical trials, would have a major impact in cost and time reduction on the clinical approval of new medicines.

Up to now, numerous patient-specific iPSC lines have been established and used for disease modelling. These are expected to simplify the approach of rare disease studies (65). To use iPSCs seems to be the better option, in opposition to conventional ways, to test for toxicology and drug research, providing a better imitation of the human physiological environment and a safer alternative (102).

iPSCs application in drug screening is also a reality. Using hepatic cells derived from iPSCs generated from patients with alpha-1 anti-trypsin (AAT) deficiency, Choi *et al.* initially screened 3131 compounds in the Johns Hopkins Drug Library identified as suited for AAT-deficiency therapy. Of that initial number 43 had the necessary FDA and international approval and were tested in 4 different AAT-deficient iPSC lines, resulting in a final count of 5 trial successful compounds.(103) Technology of iPSCs is also valuable for ADME (Absorption, Distribution, Metabolism, Excretion) studies, having had an important role in drug discovery (104).

Such outcomes not only demonstrated that the recreation of disease phenotypes using patient derived iPSCs was possible, but also the potential applications of iPSCs in drug screening including drug readjustment.

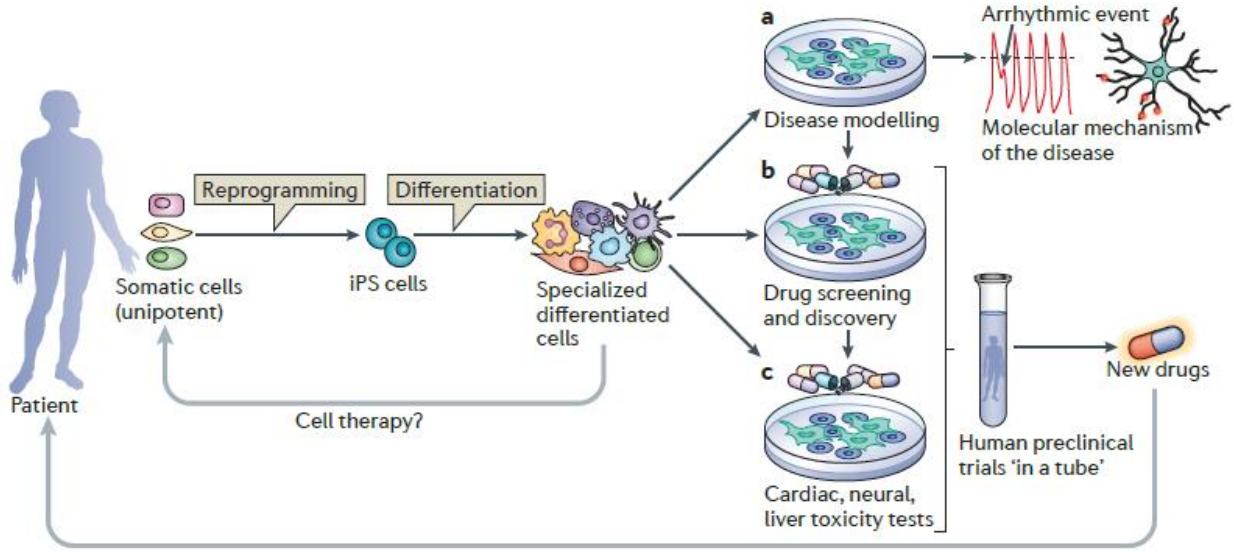

Figure 1 - Human iPSC derivation, differentiation and applications. Adult somatic cells (unipotent can be reprogrammed into iPSCs. After *in vitro* induced differentiation, human iPSCs form specialized cells that have various applications. **a |** Human iPSC may be used in disease modelling to understand the molecular mechanisms underlying disease phenotypes. **b |** Application of human iPSCs is in drug screening and research, to determine the effects of candidate medicines and new compounds and identify target pathways. **c |** Human iPSC are also valuable in cardiac, neural and liver toxicity tests to assess cellular toxic responses. Drug screening and toxicity tests together represent human preclinical 'trials in a tube' that allow the introduction of 'the patient' in early stages of the drug discovery process. Adapted from: (62)

4.6 iPSC-based regenerative therapy

One of the most appealing features of iPSCs technology is the opportunity it offers to generate autologous cells to use in cell-replacement therapy. The potential to promote damaged tissues replacement and development of endogenous regenerative mechanisms make iPSCs extremely advantageous.

In regenerative medicine, the tissues that are injured or in degeneration can be repaired using differentiated cells, originated from iPSCs derived from somatic cells of the own patient, that will then be transplanted into the specific site of damage in the patient's body. This iPSC-based autologous methodology has plenty of benefits when compared with allografts from other donors that comprise a risk of immunological rejection and infection by unidentified viruses or other microorganisms. The fact that the availability of donors is so low and the hurdle it is to find organs with a perfect physiological profile match which check all the health parameters and tests that need to be done before a transplant, make iPSCs a better approach (77).

Gene therapy with resort to iPSCs is also starting to be an explored field, as a result of the growing technology of genome editing, including for example Zinc Finger Nucleases (ZFN), CRISPR/Cas systems, among others. This way it is possible to insert or delete whole genes or nucleotides, for disease modelling, which has already been tried for the treatment of degenerative diseases symptoms, or treatment of various primary immunodeficiencies like *Wiskott-Aldrich syndrome* (WAS), an X-linked deficiency caused by mutation in WAS gene(105). When the desired specific cells are formed, they will then be transplanted to a specific site. In case it is a disease originated by a mutation, such mutation will first be corrected to form a normal iPSCs and only then be differentiated into the specific wanted cells (77).

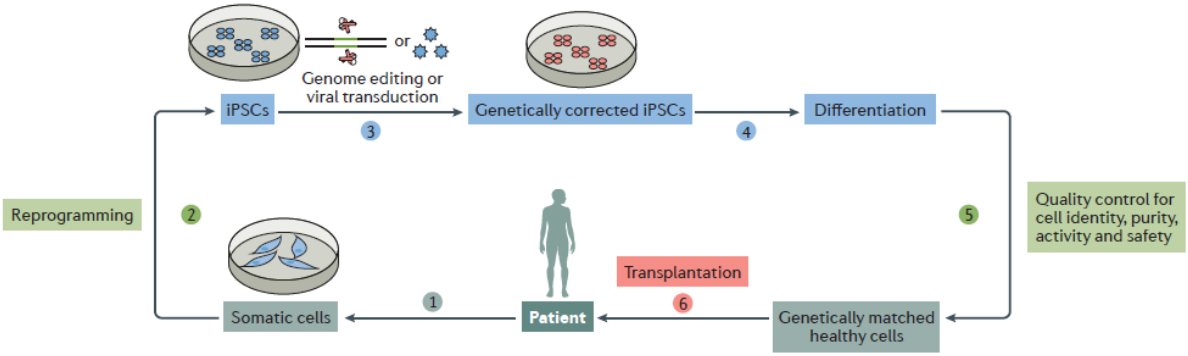

Figure 2 - Human iPSC-based cell therapy. There are six main steps that can be considered: 1. Somatic cells are collected from affected patients and cultured. 2. Patients' somatic cells are reprogrammed into iPSCs. 3. Genetic correction of the patient-derived iPSCs occurs. 4. The corrected iPSCs are differentiated into desired cell types to serve as genetically matched healthy donor cells. 5. Quality control tests for cell identity, purity, activity and safety are performed. 6. Genetically matched healthy cells are transplanted into patients: cell therapy. Adapted from: (63)

Despite the promise of iPSCs in cell therapy, there are several challenges that still need to be addressed. One example is the concern of tumorgenicity, which implies very demanding regulatory requirements for its approval in clinical trials. (106)

The capacity to enable a cell-based therapy with iPSCs depends, ultimately, on the "efficiency of cell-lineage-specific differentiation, efficiency of cell purification to eliminate the risk of teratoma, and development of novel cell delivery methods to introduce cells of interest into relevant organs" (106).

The first clinical trial, where iPSCs were aimed to be used in cell therapy, was launched in 2014, by the team of Masayo Takahashi, an ophthalmologist at the RIKEN Center for Developmental Biology (CDB) in Kobe, Japan. It was in 2012 when Takahashi was developing ESC-based treatments for retinal diseases that Yamanaka first published his method for reprogramming iPSCs (107). With such discovery, she quickly decided to change her research to iPSCs, and eventually began to collaborate

with Yamanaka. By 2013, they succeeded in the creation of sheets of retinal pigment epithelium (RPE) cells, from iPSCs that were previously derived from skin cells of two people with age-related macular degeneration, an eye condition that can generate blindness. Finally, in 2014 they initiated a transplant into the eye of a women, with her own cells, beginning the first clinical study using human iPSC products. The therapy had a favourable outcome, with the improvement of the patient's vision and interruption of her macular degeneration. Even though it had such a successful result, the trial was put on hold after two genetic changes were detected in both the patient's iPSCs and the RPE cells derived from them. There was no evidence that those mutations were related to tumour formation, but they decided the safest way was to interrupt the trial, with prevision to resume (108,109).

Early Phase I and Phase II clinical trials are experimenting the therapeutic interest and security of iPSC-based cell treatment in main fields with clinical gaps, such as: neurodegenerative diseases, spinal cord injuries, heart conditions, diabetes, hematopoietic disorders (recently there has been accumulating data on the use of iPSCs for *ex-vivo* blood expansion of a variety of blood elements), liver damage, among others (110–113).

Even though a good number of clinical trials are now evolving for iPSC derivatives, it will take many years to work out a suitable method for making the right cell types, in large enough quantity and adequate purity, before they are available for patient use. The safety of iPSCs for regenerative purposes implies a variety of preclinical assays, including measuring chromosomal stability, searching for mutation in oncogenes and housekeeping genes, as well as genes that are more expectable to affect the function of the cell. The problem is the insufficient agreement on what standards should be used to do such assays, admitting a common understanding of the importance of this testing (108,110).

As stated by Robert Lanza, chief scientific officer in Astellas Institute for Regenerative Medicine in Marlborough, USA, "*iPS cells are the most complex and dynamic therapies that have ever been proposed for the clinic. I'm the first one who wants to see these cells in the clinic, but an abundance of caution is needed*."(109)

5. Use of iPSCs in Neurodegenerative diseases

The human brain is made of billions of neurons and glial cells that form an elaborate circuit pattern. The central nervous system is one of the most fragile elements of the body and has a limited capacity of regeneration (8).

Neurodegenerative diseases are defined by a gradual loss of neuronal subtypes in the brain and spinal cord, associated with the presence of aberrant protein inclusions that can result from mutations in disease-related gene(s), being also the consequence of ageing and environmental factors. Among this diseases the number of familial and sporadic cases varies, with the underlying genetic not always matching the same phenotype or pathology (114,115).

Therefore, there is an attempt to formulate extensive patient-derived cell line cohorts and integrative phenotyping pipelines to determine significant targets for therapeutics. Gene mutation-positive iPSCs have demonstrated they are able to reproduce disease-relevant phenotypes, as verified by *post-mortem* pathology and biomarkers, giving understanding into early disease mechanisms and common progression of neurodegeneration in such diseases (116–118).

As discussed in previous topics, human iPSCs can be converted into the cell types of interest. Creation of functionally specialized neural subtypes relies on manipulation of cultures in the presence of definite factors which promote the conversion of pluripotent cells into neural progenitors, neurons, and glia. Various protocols have been established, however the development of iPSC-derived models for late on-set neurodegenerative diseases has been a challenge due to the derived in vitro neuron's immaturity. Presently, two-dimensional (2D) or three-dimensional (3D) methodologies of neural differentiation have presented incredible potential for generation of suitable cultures (53,117,118).

Current investigations indicate that the transcriptional and electrophysiological properties of iPSC-derived neurons are more similar to fetal neurons than adult's. It is possible that external factors, which are present during normal development or ageing, are necessary to activate the maturation process of these cells. Hence, researchers are exploring ways to stress cells or introduce proteins that age them prematurely, taking in consideration that age is the strongest risk factor for neurodegenerative diseases (119,120).

Recently, human iPSCs-patient derived, from neurodegenerative diseases, including AD, PD and ALS, have had success in their *in vitro* differentiation into motor neurons, dopaminergic neurons and oligodendrocytes, among others (119–121). Even though there are multiple challenges to be faced, iPSCs use for transplantation purposes could be an important alternative strategy to treat patients with neurodegenerative diseases. The main goal would be to produce new neurons to

replace the lost or non-functioning ones during disease progression, or to generate glial cells to protect neurons from continuing degeneration.

5.1 iPSCs in Alzheimer's Disease

The pathogenesis of AD has been intensively studied in the last decade and iPSCs have been widely used to investigate both familial and sporadic forms of the disease. However, modelling sporadic diseases using iPSCs is possibly more challenging, due to the phenotypic changes being often induced by non-patterned small-effect genetic risk variants in combination with environmental factors (66).

Regardless of the complex genetic background of AD, there have been successful AD-iPSCs models, including not only neurons, but other cell types, from 2D to 3D and chimeric models.

Israel *et al.* (122), in 2012, were the first to determine AD mechanisms *in vitro* using iPSC-derived neurons from patients with familial (APP genetic duplication) and sporadic AD, fAD and sAD, respectively. They reported elevated levels of Aβ40 and Tau phosphorylation in the iPSC-derived neurons from fAD and sAD subjects, when compared with neurons with absence of the disease (122). One other key phenotypic AD element that was successfully reproduced in many studies is the altered Aβ40/ Aβ42 ratios and Aβ aggregation, using iPSCs-based modelling of fAD mutations in APP, PSEN1, PSEN2 and APOE genes (123–129). It was also reported that pTau is found in βD-iPSC-derived neurons with PSEN1 mutations (123,130).

A more recent approach has been the three-dimensional (3D) neural cell culture models, organoid culture systems, which have allowed the investigation of complex cellular networks and replicate clinically-observed AD pathologies, for example, Aβ aggregation or higher Aβ42 levels. This means that 3D models are similar to what is observed in the *in vivo* iPSCs models or clinical cases of AD (131–133).

Furthermore, human iPSC/mouse chimeric models, which consist on the transplantation of patient-specific iPSC-derived neurons into the brains of AD affected mice have proven to be a patient-specific way to model the disease in a seemingly physiological, three-dimensional environment. Espuny-Camacho *et al.* developed a novel chimeric model for AD, demonstrating human-specific pathological features, highlighting its relevance (134).

In terms of therapeutic findings, various anti-amyloid drugs targeting different pathways of Aβ42 production and/or aggregation have been developed and started

clinical trials. A research group developed a model using a plant polyphenol, apigenin, to study its anti-inflammatory and neuroprotective properties in AD-patients derived neurons. This study showed that apigenin molecules were able to promote global down-regulation of cytokine and nitric oxide (NO) release in inflammatory cells and protect iPSC-derived AD neurons against apoptosis (135). Some other research from Brownjohn *et al*. consisted on a designed phenotypic small-molecule screen to identify modulators of APP processing that would increase the relative production of short Aβ42 peptides, nontoxic forms, in human trisomy 21/Down syndrome neurons, a complex genetic model of AD. This study identified anthelmintic compounds, avermectins, that showed a secretase-independent indirect way of modulating APP processing (136). Resorting to human iPSC-derived AD model neurons, another research group observed that nobiletin (natural compound from citrus peel) could enhance the degradation of intra- and extracellular Aβ levels, meaning that this compound could represent a promising novel prophylactic seed medicine, or functional food for AD (137).

A study from Bright *et al.* used patient-derived iPSC model of AD and identified a relevant protein, extracellular Tau (eTau), from AD patient derived cortical neuron conditioned media. They were able to generate a therapeutic antibody against eTau, based on a comparison between AD patients neurons and age-matched controls, and also postulate a connection between tau and Aβ, suggesting a dynamic mechanism of positive feed forward regulation (138). With such investigation this research group was able to develop BMS-986168, a specific antibody for the Tau fragments. It was later in 2017, that BIIB092 (formerly known as BMS-986168), licensed by Biogen, entered a Phase II clinical trial for AD and supranuclear palsy.

The advantage of the 3D culture system is shown by Raja *et al.* in a study where they created organoids from multiple fAD patient iPSC lines and were able to achieve relevant AD-like phenotypes. Those organoids were then treated with two compounds, known to reduce amyloid aggregation: the γ-secretase inhibitor Compound E (Comp-E; γ2) and a BACE-1 β-secretase inhibitor. Such treatment was able to significantly reduce the number of amyloid aggregates in the fAD organoids. Those results were consistent with the concept that 3D iPSC-derived organoid is appropriate to compound testing and offer great potential to increase the translatability of pre-clinical drug discovery in AD (131).

Several studies are underway to unravel the role of iPSCs in AD as described in Table 2 and Table 3.

One recent use of IPSC technology, directed to Alzheimer's treatment, has been the iPSC-derived macrophages expressing Neprilysin-2 (NEP2), which is a protease with Aβ-degrading activity. In that study, the IPS-macrophages were transplanted into an *in vivo* mouse model of AD, demonstrating to be successful at degrading toxic β-amyloid deposits. These macrophages, genetically altered and derived from iPSC, represent a potential treatment for AD (139).

In regard to cell replacement therapy for AD, the potential is enormous, but it is still a challenging area. Fujiwara *et al.* conducted a transplantation of neuronal precursors of cholinergic neuron phenotype, derived from human iPSC, into the bilateral hippocampus of AD mice, and discovered that the spatial memory was significantly improved (140). In addition, Tong *et al.* transplanted embryonic interneuron progenitors into aged apoE4 knock-in mice, discovering that the transplanted cells developed into mature interneurons, functionally integrated into the hippocampal circuitry, and restored normal learning and memory (141). Such preclinical advances demonstrate that transplanted human iPSCs-derived cells are able to truthfully rescue pathological alterations of AD patients, representing 'proof-of-concept' for the clinical translation of iPSCs to cell-therapy of AD.

Table 2 - Patient-derived iPSC-based modelling AD.

Cell type	Mutation origin	Genetic Deffect	Target Form	Phenotypes	References
Neurons	Patients	APP Dp	fAD sAD	↑ Aβ40 secretion ↑ pTau ↑ active GSK3β ↑ number of endosomes	Israel *et al.* (122)
Forebrain neurons	Patients	APP (V717I)	fAD	↑ Aβ42/Aβ40 ratio altered APP subcellular localization ↑ tTau and pTau	Muratore *et al.* (123)
Neurons	Patients	PSEN1 (A246E)	fAD	↑ Aβ42/Aβ40 ratio, ↑ expression of FOXG1, mGluR1 and SYT1	Mahairaki *et al.* (127)
Neurons	Patients	PSEN1 (A246E) PSEN2 (N141I)	fAD	↑ Aβ42 secretion	Yagi *et al.* (126)
Forebrain neurons	Patients	PSEN1 (Y115C, M146I) APP (V717I) APP Dp	fAD	↑ Aβ42 secretion, ↑ intracellular tTau and pTau	Moore *et al.* (130)
Organoid	Patients	APP Dp PSEN1 (M146I) PSEN1 (A264E)	fAD	↑ levels of Aβ ↑ Aβ aggregates ↑ pTau abnormal endosome morphology and recycling	Raja *et al.* (131)
Forebrain Neurons	Patients	APOE (E3/E4)	sAD	↑ Aβ42/Aβ40 ratio ↑ vulnerability to glutamate-mediated cell death	Duan *et al.* (128)
Cortical Neurons	CRISPR/Cas9 system	APP (Swe) PSEN1 (M146V)	fAD (Early onset)	↑ Aβ levels and Aβ42:40 ratios	Paquet *et al.* (125)

Table 3 - iPSC-based AD therapies research.

Model	Screening Strategy	Experimental molecule(s)/therapy	References
AD Neurons PSEN1 (P117R) (APOE3/3); (APOE4/4); (APOE3/4)	Down-regulation of cytokine NO release in inflammatory cells; ↑ frequency of Ca2+ signals, caspase-3/7 mediated apoptosis.	Apigenin	Balez *et al.* (135)
AD cortical neurons (TS21)	Secretase-independent indirect way of modulating APP processing: ↑ short Aβpeptides ↓Aβ42/38	Avermectins	Brownjohn *et al.* (136)
AD neurons (PSEN1 P117L)	Degradation of intra- and extracellular Aβ levels	Nobiletin	Kimura *et al.* (137)
AD neurons (PSEN1-A260V) (PSEN1-L286V) (PSEN1-L418F) (PSEN2- N141I)	Inhibition spread of tau; ↓ CNS Aβ levels; neuronal hyperactivity.	BIIB092 anttibody anti eTau Phase II clinical trial (Biogen)	Bright *et al.* (138)
AD Organoid APP Dp, PSEN1 (M146I) PSEN1 (A264E)	↓ the number of amyloid aggregates	γ-secretase inhibitor Compound E (Comp-E; γ2); BACE-1 β-secretase inhibitor.	Raja *et al.* (131)
In vivo Mouse model of AD	Degrading toxic β-amyloid deposits	iPS-macrophages-like (ML) expressing NEP2	Takamatsu *et al.* (139)
In vivo Mouse model of AD	Spatial memory significantly improved	<u>Regenerative therapy:</u> Neuronal precursors with cholinergic neuron phenotype (hiPSC-derived)	Fujiwara *et al.* (140)
In vivo Mouse model of AD	Restored normal cognitive function: learning and memory	<u>Regenerative therapy:</u> Embryonic medial ganglionic eminence (MGE)-derived interneuron progenitors	Tong *et al.* (141)

5.2 iPSCs in Amyotrophic Lateral Sclerosis

ALS is a progressive neurodegenerative disease characterized by cortical and spinal motor neurons loss, which leads to muscle atrophy and paralysis, ending in death (33).

Despite the growth in the understanding of ALS pathogenesis, with the use of transgenic animal models, there are, so far, no therapies capable of providing a significant clinical benefit for ALS patients, meaning ALS remains an incurable disease (142). In order to explore the mechanisms that lead to motor neurons degeneration and discover new therapies, there is a need to have a better insight of ALS neuropathology, as the underlying mechanisms of protein accumulation and how it leads to selective degradation of motor neurons are still, mostly, unknown (33,142).

. Human-derived iPSCs models provide a way to test *in vitro* some pathogenetic theories of ALS and study early disease mechanisms using patient cells. For the first time, in 2008, Dimos *et al.* showed it was possible to differentiate patient-specific iPSCs (skin fibroblasts) into motor neurons. They were able to generate iPSCs from an 82-year-old woman, diagnosed with a familial form of ALS, giving hope that such cells could be used for ALS modelling, drug discovery, and eventually autologous cell replacement therapies (143). Since then, some studies have reported the differentiation of iPSC from ALS patients into motor neurons (144–150).

Burkhardt *et al.*, using iPSCs derived from patients with the sporadic form of ALS, were able to identify *de novo* aggregation of TAR DNA-binding protein 43 (TDP43) in the patients' motor neurons and then use such phenotype to arrange a chemical screen using the TDP-43 aggregate endpoint. The goal was to identify compounds that reduced the TDP43 aggregation and the results were that some small molecule modulators such as cyclin-dependent kinase inhibitors (CDK), c-Jun N-terminal kinase inhibitors (JNK), Triptolide, and some cardiac glycosides (such as digoxin), demonstrated such effect (150).

Focusing on the superoxide dismutase (SOD1) gene mutation, responsible for 20% of the total of cases of familial ALS (148), Popescu *et al.* demonstrated that human iPSC-derived neural progenitor cells can be successfully transplanted into ALS-like environments, *in vivo,* and continue to differentiate and survive as human mature neurons (146).

Some other researches have used iPSCs model to study the hyperexcitability phenotype that is widely related to many ALS mutations (149,151). Wainger *et al.,* using iPSC-derived motor neurons from ALS patients, which were found to be hyperexcitable compared to controls, discovered that Kv7 channel activator (potassium channel agonist) ezogabine/retigabine, an approved drug for epilepsy, could block the hyperexcitability and improve motor neuron survival *in vitro* (149). At this day, ezogabine has finished a placebo-control Phase II clinical trial, with 192 ALS patients in association with *GlaxoSmithKline.* The trial achieved its primary goal of measuring a reduction in motor neuron excitability in people with ALS following treatment. Lucie Bruijn, PhD, MBA, chief scientist, The ALS Association, claimed "*This is the first clinical trial for ALS that was designed using data based on an iPSC model of ALS and was possible in part due to the availability of a biomarker in people living with disease that measures excitability of motor neurons, also characterized in the iPSC model*." (152)

The use of IPSC for transplantation purpose in ALS, has already shown some potential, as described in the following Tables 4 and 5. In a study from Nizzardo *et al.*, iPSC-derived neural cells were found to promote a better neuromuscular function and increase lifespan. These positive effects are linked to multiple mechanisms, such as the production of neurotrophic factors and reduction of microgliosis(153).

Table 4 - Patient-derived iPSC-based modelling ALS.

Cell type	Genetic Deffect	Phenotypes	References
Motor Neurons	SOD1 (L144F)	-	Dimos et al. (143)
Motor Neurons	ALS8 vamp-associated protein B/C (VAPB) gene mutation (P56S)	↓VAPB protein levels	Mitne-Neto *et al.* (144)
Motor neurons	TDP-43 mutations: (Q343R, M337V, G298S)	Cytosolic aggregates of TDP-43 and shorter neurites.	Egawa *et al.* (145)
Motor neurons	Mild or severe mutations in the FUS gene	↑ Susceptibility to cell stress and FUS mislocalization (neurites and cytoplasm)	Higelin *et al.* (147)
Motor neurons	SOD1A4V mutation (SOD1+/A4V)	↑ oxidative stress, ↓ mitochondrial function, altered subcellular transport, activation of the ER stress and unfolded protein response pathways.	Kiskinis *et al.* (148)
Motor neurons	SOD1 Mutation (SOD1+/A4V)	Hyperexcitability	Wainger *et al.* (149)
Motor neurons	SOD1 and FUS mutations	TDP-43 aggregation	Burkhardt *et al.* (150)

Table 5 - iPSC-based ALS therapies research.

Model	Screening Strategy	Experimental molecule(s)/therapy	References
ALS motor neurons (SOD1 mut) (FUS mut)	↓ TDP43 aggregation	CDK inhibitors JNK inhibitors Triptolide Cardiac glycosides	Burkhardt *et al.* (150)
In vivo Mouse model of ALS	Survival and differentiation into motor neurons, of human iPSC-derived neural progenitors	Regenerative therapy: Survival and differentiation of human iPSC-derived neural progenitors	Popescu *et al.* (146)
ALS motor neurons (SOD1A4V/+)	Rescue of hyperexcitability	Ezogabine/retigabine **Phase II clinical trial** (GlaxoSmithKline)	Wainger *et al.* (149)
ALS Motor neurons TDP-43 **(**Q343R, M337V, and G298S)	Rescue of motor neurons phenotypes	Anacardic acid	Egawa *et al.* (145)
In vivo Mouse model of ALS	Improved neuromuscular function; Increased life span	Regenerative therapy: Neural stem cell population from human iPSCs based on high aldehyde dehydrogenase activity, low side scatter and integrin VLA4 positivity	Nizzardo *et al.* (153)

5.3 iPSCs in Parkinson's Disease

PD is a very common neurodegenerative disease, the second most common, characterized by a variety of motor an non motor symptoms. The majority of the symptoms are motor and result from the death of dopaminergic neurons in the *substantia nigra*. Therefore, that is where most of the research efforts are focusing, dopaminergic neurons (47,154).

PD cases result from both genetic and environmental factors, being defined as sporadic or familial cases, with the latter being caused by known genetic mutations, mostly in genes that are involved in the regulation of mitochondrial function and oxidative stress (6).

Human iPSCs have been extensively used to study Parkinson's pathogenesis associated with inherited monogenetic mutations, familial PD, as well as the sporadic form of PD.

Most of the studied iPSC lines of familial PD have been carrying the G2019S mutation in the LRRK2 gene, a very common mutation associated with this disease, but still with unclear function (155). Nguyen *et al*. studied this G2019S mutation in iPSC that were able to differentiate into dopaminergic neurons, demonstrating that they showed increased expression of key oxidative stress response genes, α-synuclein protein and caspase-3 activation (156). Other iPSC-based studies with the mutation in LRRK2 have been performed, suggesting its important role on the survival of neurons (157–159).

Another typical gene that is affected in familial PD is the SNCA gene, which encodes α-synuclein and has been widely studied. Even though the function of α-synuclein is not completely known, a-synuclein aggregation in Lewy bodies is a major pathological phenotype of PD (6). Devine *et al.* studied iPS cell-derived neurons in which the SNCA locus was triplicated, observing that they produced double the amount of α-synuclein protein, when compared to control neurons established from an unaffected first-degree relative.(160) A different group also generated a human iPSC-based model with a G209A (p.A53T) αSyn mutation that causes a familial form of PD characterized by early onset and a generally severe phenotype. Those iPSC-derived neurons showed the disease-relevant cellular phenotypes and were tested with small molecules targeting αSyn. Such small molecules reverted the degenerative phenotype, indicating a treatment strategy for PD and other synucleinopathies (161). Apart from these, many other studies, focusing on other gene mutations and risk factors for the

sporadic form of PD have used iPSC-based models. (162–164). Some of these can be found described in the Table 6 below.

Regarding therapeutic discoveries, iPSCs have also had an important role. In a research from Chung *et al.* iPSCs derived from PD patients were used to confirm the therapeutic effectiveness of a small molecule identified in a yeast screening, NAB2 (Nedd4 ubiquitin ligase activator). Such molecule was able to reverse pathologic phenotypes in the iPSC-derived neurons, being identified as a potential anti-PD agent (165). Another study demonstrated that GW5074 (an LRRK2 kinase inhibitor), antioxidant coenzyme Q10 and rapamycin (mTOR inhibitor), could prevent neuronal cell death, implying that blocking LRRK2 kinase activity may be a valuable drug mechanism (159).

Some other investigation came from Burbulla *et al.*, where with the use of dopaminergic neurons derived from patients with PD, both idiopathic and familial forms, a time-dependent pathological cascade was identified. It translated in "mitochondrial oxidant stress leading to oxidized dopamine accumulation and ultimately resulting in reduced glucocerebrosidase enzymatic activity, lysosomal dysfunction, and α-synuclein accumulation." (166). They were able to demonstrate that an early treatment with mitochondrial antioxidants can lower the accumulation of oxidized dopamine and α-synuclein, preventing this way the lysosomal dysfunction (166). In another recent study, from Mital *et al.* group, resorting to an impartial screen targeting endogenous gene expression, it was learnt that the β2-adrenoreceptor (β2AR) is a regulator of the α-synuclein gene (SNCA). β2AR agonists clenbuterol and salbutamol, were able to lower SNCA expression in a dose- and time-dependent manner. During a follow-up period of eleven years with a longitudinal analysis of 4 million Norwegians, salbutamol, the β2AR agonist - brain-penetrant bronchodilator medication, was associated with reduced risk of developing PD. Evaluation in additional populations and in clinical trials will be needed to determine whether these findings can be translated to patients with PD (167).

Recently there have been theories of a relation between the adaptive immune system and PD, since it was detected a superior Th17 frequency in blood and upregulated T lymphocytes in *post-mortem* tissues of the disease. In this research, after a co-culture with activated T lymphocytes, PD iPSC-derived neurons underwent higher neuronal death, via upregulation of IL-17 receptor and NF-kB activation. The blockage of IL-17R, by the IL-17 antibody, secukinumab, provided a potential solution for rescue of neuronal cell death (168). Such studies are described in the Table 7, below.

iPSC technology has been expected to fit the purpose of cell transplantation, regenerative therapy. As the injection of therapeutic cells can be targeted to the *susbtancia nigra*, where dopaminergic neurons' degeneration occurs, PD is highly susceptible to the cell replacement therapy. There are, in fact, several groups eminently close to carrying their PSC-derived DA neurons to clinical trials.

Since its start in 2007 at Kyoto University, research and further applications of human iPSCs have had the support of the Japanese government. In fact, the nucleus study place for iPSCs is considered to be the Center fo iPS Cell Research and Application (CiRA), at Kyoto University (169).

In 2013, Japanese government started a project named "*Research Center Network for Realization of Regenerative Medicine*", with the main goal of encouraging a quicker research and progress for the clinical application of iPSCs, in which PD was defined as one of the target diseases.

There have already been trials of transplanting iPSC-derived neural cells into nonhuman primate brains, that were successful in demonstrating that the immune response that occurs is minimal (170,171). This means it would not be necessary to use immunosuppressant drugs upon transplantation, averting this way, adverse effects such as kidney or liver dysfunction and also there would be no risk of transmitting any type of pathogen from other persons.

Autologous transplantation is an expensive, time-consuming and laborious process, with much requirements, so, with such factors in consideration, CiRA planned to perform as an initial trial, an allogenic transplantation. After the induction and selection of the DA neurons, its function in the brain must be meticulously evaluated before proceeding to clinical application. Hence, in terms of animal studies, CiRA used not only rodents, but also monkeys as PD models (169).

A very important factor is the condition of the host brain environment, "*for a successful neuronal transplantation, the grafted cells need to survive, extend neurites, and form synapses with the host neurons*."(169) This way, medicines or gene modifications that promote cell survival, neurite extension, and synapse development would heighten the therapeutic effect of the grafted cells. In such a way, to promote the prosperity of regenerative medicine against PD it would be decisive to have am association of cell transplantation, medicines, and rehabilitation (169).

Recently, Takahashi's team announced the first human clinical trial of iPSC-generated dopaminergic progenitor cells transplantation into PD patients. This trial began in August 2018 at Kyoto University Hospital and even though the cells are HLA

matched, patients still received immunosuppressant therapy. Nearly 5 million cells were administered through two drilled holes in the skull of 7 moderate PD patients. The progression of the disease, as well as other side effects, will be monitored, with the future of this iPSCs- based PD therapy seeking to be highly promising.(172,173)

Table 6 - Patient-derived iPSC-based modelling PD.

Cell type	Genetic Deffect	Phenotypes	References
Dopaminergic Neurons	LRRK2 mutation (G2019S)	↑ expression of key oxidative stress response genes; ↑ α-synuclein protein; ↑ sensitivity to stress-induced cell death.	Nguyen *et al.* (156)
		↓ of neurites; neurite arborization; accumulation of autophagic vacuoles.	Sanchez-Dane's *et al.* (158)
Human neural stem cells		↑susceptibility to proteasomal stress; ↑ passage-dependent deficiencies in clonal expansion and neuronal differentiation.	Liu *et al.* (157)
Dopaminergic Neurons	Triplication of SNCA	Double the amount of α-synuclein protein produced.	Devine *et al.* (160)
Neurons	SNCA mutation (G209A)	↑ protein aggregation; compromised neuritic outgrowth; contorted or fragmented axons; swollen varicosities containing αSyn and Tau.	Kouroupi *et al.* (161)
Dopaminergic Neurons	Glucocerebrosidase (GBA) mutation (N370S)	↑ α-synuclein levels; ↓ dopamine levels; Induced MAOB expression; Disrupted network activity.	Woodard *et al.* (163)

Table 7 - iPSC-based PD therapies research

Model	Screening Strategy	Experimental molecule(s)/therapy	References
PD cortical neurons α-Syn (A53T)	Rescue of ER processing, ↓nitric oxide levels	NAB2	Chung *et al.* (165)
PD neural cells PINK1 (Q456X) LRRK2 (R1441C) LRRK2 (G2019S)	Rescue of cellular vulnerability associated with mitochondrial dysfunction	GW5074 (LRRK2 kinase inhibitor); antioxidant coenzyme Q10; rapamycin (mTOR inhibitor).	Cooper *et al.* (159)
PD dopaminergic neurons (idiopathic or DJ-1 c.192G>C)	Rescue of mitochondrial oxidative stress, ↓oxidized dopamine, ↓α-Syn, ↑lysosomal function	Mitochondrial antioxidants: FK506; Isradipine; Mito-TEMPO; N-acetylcysteine.	Burbulla *et al.* (166)
PD neurons SNCA locus tripplication	↓SNCA expression and rescue of oxidative stress	Clenbuterol	Mittal *et al.* (167)
Sporadic PD midbrain neurons co-culture with Th17 lymphocytes	Rescue of the T lymphocyte-induced neuronal cell death by blockage of either IL-17 or IL-17R,	Secukinumab (IL-17 antibody)	Sommer *et al.* (168)
In vivo Primate PD Model	↑ in spontaneous movement of the monkeys; Extended dense neurites into the host striatum	<u>Regenerative therapy:</u> Mature dopaminergic neurons	Kikuchi *et al.* (170)
In vivo **Clinical Trial**	Basic Objectives: Safety, Efficacy	<u>Regeneartive therapy:</u> Dopaminergic progenitors	(173)

6. Challenges of iPSC technology

In spite of the great potential iPSC-derived neurons represent and the several advantages, there are still many challenges to overcome.

One of the preoccupations is the diversity of iPSC characteristics, which has been explained in various ways such as it being a retained epigenetic memory, genetic background, or as it being newly obtained features during reprogramming (174,175). Further studies to discover molecular markers to evaluate iPSC quality are required in the future.

The reproduction of iPSCs with the use of retroviral or lentiviral systems imply a concern related to the incorporation of the viral system in the host genome, as the genetic material might integrate randomly and generate genetic aberrations and teratoma formation, with the oncogene reactivation (5,107). Zhang *et al.* stated that out of 593 genes that are expressed in iPSCs, 209 genes were found to be expressed in cancer tissues and tumour cells (176). Aside from the safety issue, the genetic and epigenetic condition of clones may alter after reprogramming and even though, the non-integrating approaches significantly reduce these risks, there is a need of researchers need for serious caution to select stable lineages for differentiation studies (2,79).

Another difficulty relates to the fact that most of high prevalence neurodegenerative diseases concerns genetic mutations that are not known or have an unidentified genetic component in combination with environmental factors. This way, it is a challenge to generate iPSC that recapitulate relevant disease phenotypes. Notably, the age factor, which is considered to be related to most of the neurodegenerative diseases and contribute to its development, is complicated to reflect it in the iPSC-based models. The amount of treatments that are added to accelerate disease phenotype expression in the iPSC, might lead to a conception of cells that do not truly reflect the cellular responses to compounds that the body would have at a physiological level and decrease its accuracy (2). In addition, to identify the correlation between early aberrant phenomenon observed from iPSC-derived pathogenic neurons and normal neuronal degeneration in the patient brain is also a big challenge (7).

Lastly, in the current status, the lack of a high efficiency conversion of the methods and the fact it is a very laborious and time-consuming process. The average conversion for different reprogramming methods is less than 1%, meaning that efficiency need to be improved in the upcoming protocols to increase the possibility for success (4).

7. Conclusion

Being such an innovative discovery, much has already been learnt and explored about iPSCs applications and the impact it can have on the therapeutics of neurodegenerative diseases. There are numerous advantages, with the most relevant relying on the fact that iPSCs are a chance for a personalized treatment, with much less ethical issues, when comparing to ESC.

Since their discovery in the early-2000s, an improved generation of iPSC lines have been created, with less risk of tumorgenicity, thus considered to be a safer approach. For the continuous success of these experiments it will be critical to uncover the molecular mechanisms underlying the reprogramming events when generating iPSCs, focusing on the safety of the process. Escalating the reprogramming efficiency without a need for genetically modification of the cells are key goals for the future of this technology.

It seems that in terms of disease modelling iPSCs have had exceptional success, contributing for great revelations in various diseases. Recently there has been an emergence of new clinical trials, showing the evolving state that this technology has had since its discovery. When it comes to neurodegenerative diseases, PD is probably the one which benefits most from iPSCs and the closest to use it for cell replacement therapy.

Another important concept is the granting of an open sharing access, where results, publications, compounds and even clinical trials results would be of access to the public. Such system of “Open Science” could be a strategy to construct an efficient foundation to support the investigation of existing data and biological sample resources, meaning it would make it not only easier but less time-consuming, to make new discoveries in the neurodegenerative disease field.

Despite all the potential and excitement linked to iPSCs technology, there still has to be much patience and persistence, with focus on the reality of the challenges that need to be faced. As Edward Stevens, research fellow at the Pfizer Neuroscience and Pain Research Unit in Cambridge, UK, says “*There’s no magic. With iPS cells or any new technology, it still takes a long time*”.(109)

8. References

1. Takahashi K, Ohnuki M. Present and future challenges of induced pluripotent stem cells. Philos Trans R Soc B Biol Sci. 2015;370:20140367.
2. Xie YZ, Zhang RX. Neurodegenerative diseases in a dish: the promise of iPSC technology in disease modeling and therapeutic discovery. Neurol Sci. 2014;36(1):21–7.
3. Yoshihara M, Hayashizaki Y, Murakawa Y. Genomic Instability of iPSCs: Challenges Towards Their Clinical Applications. Stem Cell Rev Reports. 2017;13(1):7–16.
4. Robinton D, Dailey J. The promise of induced pluripotent stem cells in research and therapy.pdf. Nature. 2013;481(7381):295–305.
5. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006;126(4):663–76.
6. Wu YY, Chiu FL, Yeh CS, Kuo HC. Opportunities and challenges for the use of induced pluripotent stem cells in modelling neurodegenerative disease. Open Biol. 2019;9(1).
7. Chang CY, Ting HC, Liu CA, Su HL, Chiou TW, Harn HJ, et al. Induced Pluripotent Stem Cells: A Powerful Neurodegenerative Disease Modeling Tool for Mechanism Study and Drug Discovery. Cell Transplant. 2018;27(11):1588–602.
8. Pen AE, Jensen UB. Current status of treating neurodegenerative disease with induced pluripotent stem cells. Acta Neurol Scand. 2017;135(1):57–72.
9. Wang H. Modeling Neurological Diseases With Human Brain Organoids. Front Synaptic Neurosci. 2018;10(June):1–14.
10. Gitler AD, Dhillon P, Shorter J. Neurodegenerative disease: models, mechanisms, and a new hope. Dis Model Mech. 2017;10(5):499–502.
11. Erkkinen MG, Kim M, Geschwind MD. Clinical Neurology and Epidemiology of the Major Neurodegenerative Diseases. Cold Spring Harb Perspect Biol. 2018;10(4).
12. Katsnelson A, De Strooper B, Zoghbi HY. Neurodegeneration: From cellular concepts to clinical applications. Sci Transl Med. 2016;8(364):1–6.

13. Wittchen HU, Jacobi F, Rehm J, Gustavsson A, Svensson M, Jönsson B, et al. The size and burden of mental disorders and other disorders of the brain in Europe 2010. Eur Neuropsychopharmacol. 2011;21(9):655–79.

14. Gustavsson A, Svensson M, Jacobi F, Allgulander C, Alonso J, Beghi E, et al. Cost of disorders of the brain in Europe 2010. Eur Neuropsychopharmacol. 2011;21(10):718–79.

15. Vardi G, Merrick J. Neurological Disorders: Public Health Challenges. J Policy Pract Intellect Disabil. 2008;5(1):75–75.

16. Olesen J, Gustavsson A, Svensson M, Wittchen HU, Jönsson B. The economic cost of brain disorders in Europe. Eur J Neurol. 2012;19(1):155–62.

17. Reitz C, Brayne C, Mayeux R. Epidemiology of Alzheimer Disease: Abstract and Introduction. Nat Rview Neurol. 2012;7(3):1–18.

18. Masters C, Bateman R, Blennow K, Towe CC, Sperling RA, Cummings JL. Alzheimer's disease. Nature. 2015;1:1–18.

19. Serrano-Pozo A, Frosch MP, Masliah E, Hyman BT. Neuropathological alterations in Alzheimer disease. Cold Spring Harb Perspect Med. 2011;1(1):1–23.

20. Holtzman DM, Bales KR, Paul SM, DeMattos RB. Aβ immunization and anti-Aβ antibodies: Potential therapies for the prevention and treatment of Alzheimer's disease. Adv Drug Deliv Rev. 2002;54(12):1603–13.

21. Artiga MJ, Bullido MJ, Sastre I, Recuero M, García MA, Aldudo J, et al. Allelic polymorphisms in the transcriptional regulatory region of apolipoprotein E gene. FEBS Lett. 1998;421(2):105–8.

22. Ryman DC, Acosta-Baena N, Aisen PS, Bird T, Danek A, Fox NC, et al. Symptom onset in autosomal dominant Alzheimer disease. Neurology. 2014;83(3):253–60.

23. Gupta S, Banerjee P, Laferla FM, Selkoe DJ. Alzheimer's Disease : Genes, Proteins, and Therapy. Physiol Rev. 2001;81(2):741–66.

24. Ashford JW. APOE genotype effects on Alzheimer's disease onset and epidemiology. J Mol Neurosci. 2004;23(3):157–65.

25. Prince M, Wimo A, Guerchet M, Ali GC, Wu YT, Prina M. World Alzheimer Report 2015. London, UK. Alzheimer's Dis Int. 2015;1–92.

26. World Health Organization., International. AD. Dementia: A public health

priority. Geneva: © World Health Organization 2012; 2012. 112 p.

27. Sloane PD, Zimmerman S, Suchindran C, Reed P, Wang L, Boustani M, et al. The Public Health Impact of Alzheimer's Disease, 2000–2050: Potential Implication of Treatment Advances. Annu Rev Public Health. 2002;23(1):213–31.

28. Brookmeyer R, Johnson E, Ziegler-Graham K, Arrighi HM. Forecasting the global burden of Alzheimer's disease. Alzheimer's Dement. 2007;3(3):186–91.

29. Daviglus ML, Plassman BL, Pirzada A, Bell CC, Bowen PE, Burke JR, et al. Risk factors and preventive interventions for alzheimer disease: State of the science. Arch Neurol. 2011;68(9):1185–90.

30. Norton S, Matthews FE, Barnes DE, Yaffe K, Brayne C. Potential for primary prevention of Alzheimer's disease: An analysis of population-based data. Lancet Neurol. 2014;13(8):788–94.

31. Gaugler J, James B, Johnson T, Scholz K, Weuve J. 2016 Alzheimer's disease facts and figures. Alzheimer's Dement. 2016;12(4):459–509.

32. Cummings JL, Morstorf T, Zhong K. Alzheimer's disease drug-development pipeline: few candidates, frequent failures. Alzheimers Res Ther. 2014;1–7.

33. Hardiman O, Al-chalabi A, Chio A, Corr EM, Robberecht W, Shaw PJ, et al. Amyotrophic lateral sclerosis. Nat Rev Dis Prim. 2017;3(17071).

34. Myszczynska M, Ferraiuolo L. New in vitro models to study amyotrophic lateral sclerosis. Brain Pathol. 2016;26(2):258–65.

35. Csobonyeiova M, Polak S, Nicodemou A, Danisovic L. Induced Pluripotent Stem Cells in modeling and cell-based therapy. 2017;649–57.

36. Ferraiuolo L. The non-cell-autonomous component of ALS: New in vitro models and future challenges. Biochem Soc Trans. 2014;42(5):1270–4.

37. Logroscino G, Traynor BJ, Hardiman O, Chió A, Mitchell D, Swingler RJ, et al. Incidence of amyotrophic lateral sclerosis in Europe. J Neurol Neurosurg Psychiatry. 2010;81(4):385–90.

38. Chiò A, Battistini S, Calvo A, Caponnetto C, Conforti FL, Corbo M, et al. Genetic counselling in ALS: Facts, uncertainties and clinical suggestions. J Neurol Neurosurg Psychiatry. 2014;85(5):478–85.

39. Lacomblez L, Bensimon G, Leigh PN, Guillet P, Meininger V. Dose-ranging study of riluzole in amyotrophic lateral sclerosis. Lancet. 1996;347(9013):1425–

31.

40. Arnerić SP, Kern VD, Stephenson DT. Regulatory-accepted drug development tools are needed to accelerate innovative CNS disease treatments. Vol. 151, Biochemical Pharmacology. 2018. 291–306 p.

41. Tysnes OB, Storstein A. Epidemiology of Parkinson's disease. J Neural Transm. 2017;124(8):901–5.

42. Ray Dorsey E, Elbaz A, Nichols E, Abd-Allah F, Abdelalim A, Adsuar JC, et al. Global, regional, and national burden of Parkinson's disease, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet Neurol. 2018;17(11):939–53.

43. Pezzoli G, Cereda E. Exposure to pesticides or solvents and risk of Parkinson disease. Neurology. 2013;80(22):2035–41.

44. Vlaar T, Kab S, Schwaab Y, Fréry N, Elbaz A, Moisan F. Association of Parkinson's disease with industry sectors: a French nationwide incidence study. Eur J Epidemiol. 2018;33(11):1101–11.

45. Li X, Li W, Liu G, Shen X, Tang Y. Association between cigarette smoking and Parkinson's disease: A meta-analysis. Arch Gerontol Geriatr. 2015;61(3):510–6.

46. Bellou V, Belbasis L, Tzoulaki I, Evangelou E, Ioannidis JPA. Environmental risk factors and Parkinson's disease: An umbrella review of meta-analyses. Park Relat Disord. 2016;23(2015):1–9.

47. Stoddard-Bennett T, Reijo Pera R. Treatment of Parkinson's Disease through Personalized Medicine and Induced Pluripotent Stem Cells. Cells. 2019;8(1):26.

48. Zhao P, Luo Z, Tian W, Yang J, Ibáñez DP, Huang Z, et al. Solving the puzzle of Parkinson's disease using induced pluripotent stem cells. Exp Biol Med. 2014;239(11):1421–32.

49. Braak H, Del K, Rüb U, Vos RAI De, Jansen ENH, Braak E. Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol Aging. 2003;24:197–211.

50. Martin I, Dawson VL, Dawson TM. Recent Advances in the Genetics of of Parkinson's Disease. Annu Rev Genomics Hum Genet. 2011;(12):301–27.

51. Nuytemans K, Theuns J, Cruts M, Van Broeckhoven C. Genetic etiology of Parkinson disease associated with mutations in the SNCA, PARK2, PINK1,

PARK7, and LRRK2 genes: A mutation update. Hum Mutat. 2010;31(7):763–80.

52. Han SSW, Williams LA, Eggan KC. Constructing and Deconstructing Stem Cell Models of Neurological Disease. Neuron. 2011;70(4):626–44.

53. Han C, Chaineau M, Chen CXQ, Beitel LK, Durcan TM. Open science meets stem cells: A new drug discovery approach for neurodegenerative disorders. Front Neurosci. 2018;12(FEB):1–8.

54. Kinch MS. An analysis of FDA-approved drugs for neurological disorders. Drug Discov Today. 2015;20(9):1040–3.

55. Bordoni M, Rey F, Fantini V, Pansarasa O, Di Giulio AM, Carelli S, et al. From neuronal differentiation of iPSCs to 3D neuro-organoids: Modelling and therapy of neurodegenerative diseases. Int J Mol Sci. 2018;19(12):1–13.

56. Pangalos MN, Schechter LE, Hurko O. Drug development for CNS disorders: Strategies for balancing risk and reducing attrition. Nat Rev Drug Discov. 2007;6(7):521–32.

57. Albanese S, Greco A, Auletta L, Mancini M. Mouse models of neurodegenerative disease: preclinical imaging and neurovascular component. Vol. 12, Brain Imaging and Behavior. Springer US; 2018. 1160–1196 p.

58. Liu Y, Sun JD, Song LK, Li J, Chu SF, Yuan YH, et al. Environment-contact administration of rotenone: A new rodent model of Parkinson's disease. Behav Brain Res. 2015;294:149–61.

59. Luo J, Lee SH, Vandevrede L, Qin Z, Ben Aissa M, Larson J, et al. A multifunctional therapeutic approach to disease modification in multiple familial mouse models and a novel sporadic model of Alzheimer's disease. Mol Neurodegener. 2016;11(1):1–14.

60. Becker LA, Huang B, Bieri G, Ma R, Knowles DA, Jafar-nejad P, et al. Therapeutic reduction of ataxin 2 extends lifespan and reduces pathology in TDP-43 mice. Nature. 2017;544(7650):367–71.

61. Elden AC, Kim HJ, Hart MP, Chen-Plotkin AS, Johnson BS, Fang X, et al. Ataxin-2 intermediate-length polyglutamine expansions are associated with increased risk for ALS. Nature. 2010;466(7310):1069–75.

62. Lopes FM, Bristot IJ, da Motta LL, Parsons RB, Klamt F. Mimicking Parkinson's Disease in a Dish: Merits and Pitfalls of the Most Commonly used Dopaminergic

In Vitro Models. NeuroMolecular Med. 2017;19(2–3):241–55.

63. Xicoy H, Wieringa B, Martens GJM. The SH-SY5Y cell line in Parkinson's disease research: a systematic review. Mol Neurodegener. 2017;12(1):1–11.

64. Bal-Price AK, Hogberg HT, Buzanska L, Coecke S. Relevance of in vitro neurotoxicity testing for regulatory requirements: Challenges to be considered. Neurotoxicol Teratol. 2010;32(1):36–41.

65. Bellin M, Marchetto MC, Gage FH, Mummery CL. Induced pluripotent stem cells: The new patient? Nat Rev Mol Cell Biol. 2012;13(11):713–26.

66. Shi Y, Inoue H, Wu JC, Yamanaka S. Induced pluripotent stem cell technology: A decade of progress. Nat Rev Drug Discov. 2017;16(2):115–30.

67. Liu H, Zhang S-C. Specification of neuronal and glial subtypes from human pluripotent stem cells. Cell. 2011;68(24):3995–4008.

68. Studien I DHES. Der Wert der ersten beiden Furchungszellen in der Echinodermenentwickelung Experimentelle Erzeugung von Teil und Doppelbildungen. Ztschr f Wiss Zool. 1891;(53):160–183.

69. Gardner RL. Mouse chimaeras obtained by the injection of cells into the blastocyst. Nature. 1968;220(5167):596–7.

70. Brinster RL. The effect of cells transferred into the mouse blastocyst on subsequent development. J Exp Med. 1974;140(4):1049–56.

71. Evans MJ, Kaufman MH, Martin GR, Nichols J, Smith A, Brons IGM, et al. Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature. 1981;448(7150):337–42.

72. Till JE, Mcculloch EA. A Direct Measurement of the Radiation Sensitivity of Normal Mouse Bone Marrow Cells. Radiat Res. 1961;(14):213–22.

73. Lan ML, Acharya MM, Tran KK, Bahari-Kashani J, Patel NH, Strnadel J, et al. Characterizing the Radioresponse of Pluripotent and Multipotent Human Stem Cells. PLoS One. 2012;7(12):1–9.

74. Thomson J a., Thomson J a. Embryonic Stem Cell Lines Derived from Human Blastocysts. Science (80-). 1998;282(November):1145–7.

75. Mosher JT, Pemberton TJ, Harter K, Wang C, Buzbas EO, Dvorak P, et al. Lack of population diversity in commonly used human embryonic stem-cell lines. N Engl J Med. 2010;362(2):183–5.

76. Yamanaka S. A Fresh Look at iPS Cells. Cell. 2009;137(1):13–7.

77. Singh VK, Kalsan M, Kumar N, Saini A, Chandra R. Induced pluripotent stem cells: applications in regenerative medicine, disease modeling, and drug discovery. Front Cell Dev Biol. 2015;3(2).

78. Yu J, Hu K, Smuga-otto K, Tian S, Stewart R, Igor I, et al. Human Induced Pluripotent Stem Cells Free of Vector and Transgene Sequences. Natl Inst Heal. 2009;324(5928):797–801.

79. Warren L, Manos PD, Ahfeldt T, Loh Y, Li H, Daley Q, et al. Highly efficient reprogramming to pluripotency and directed differentiation of human cells using synthetic modified mRNA. Natl Inst Heal. 2010;7(5):618–30.

80. Miyoshi N, Ishii H, Nagano H, Haraguchi N, Dewi DL, Kano Y, et al. Reprogramming of mouse and human cells to pluripotency using mature microRNAs. Cell Stem Cell. 2011;8(6):633–8.

81. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, et al. Induced pluripotent stem cell lines derived from human somatic cells. Science (80-). 2007;318(5858):1917–20.

82. Liu H, Ye Z, Kim Y, Sharkis S, Jang YY. Generation of endoderm-derived human induced pluripotent stem cells from primary hepatocytes. Hepatology. 2010;51(5):1810–9.

83. Kunisato A, Wakatsuki M, Shinba H, Ota T, Ishida I, Nagao K. Direct Generation of Induced Pluripotent Stem Cells from Human Nonmobilized Blood. Stem Cells Dev. 2011;20(1).

84. Loh YH, Agarwal S, Park IH, Urbach A, Huo H, Heffner GC, et al. Generation of induced pluripotent stem cells from human blood. Blood. 2009;113(22):5476–9.

85. Aasen T, Raya A, Barrero MJ, Garreta E, Consiglio A, Gonzalez F, et al. Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. Nat Biotechnol. 2008;26(11):1276–84.

86. Seki T, Yuasa S, Oda M, Egashira T, Yae K, Kusumoto D, et al. Generation of induced pluripotent stem cells from human terminally differentiated circulating t cells. Cell Stem Cell. 2010;7(1):11–4.

87. Song B, Niclis JC, Alikhan MA, Sakkal S, Sylvain A, Kerr PG, et al. Generation of induced pluripotent stem cells from human kidney mesangial cells. J Am Soc Nephrol. 2011;22(7):1213–20.

88. Zhou T, Benda C, Duzinger S, Huang Y, Li X, Li Y, et al. Generation of induced pluripotent stem cells from urine. Supplementary Methods. J Am Soc Nephrol. 2011;22(7):1221–8.

89. Murry CE, Keller G. Differentiation of Embryonic Stem Cells to Clinically Relevant Populations: Lessons from Embryonic Development. Cell. 2008;132(4):661–80.

90. Lee G, Papapetrou EP, Kim H, Chambers SM, Mark J, Fasano CA, et al. Modeling Pathogenesis and Treatment of Familial Dysautonomia using Patient Specific iPSCs. Nature. 2009;461(7262):402–6.

91. Matsa E, Rajamohan D, Dick E, Young L, Mellor I, Staniforth A, et al. Drug evaluation in cardiomyocytes derived from human induced pluripotent stem cells carrying a long QT syndrome type 2 mutation. Eur Heart J. 2011;32(8):952–62.

92. Hockemeyer D, Jaenisch R. Induced pluripotent stem cells meet genome editing. Cell Stem Cell. 2017;18(5):573–86.

93. Park IH, Arora N, Huo H, Maherali N, Ahfeldt T, Shimamura A, et al. Disease-Specific Induced Pluripotent Stem Cells. Cell. 2008;134(5):877–86.

94. Briggs JA, Mason EA, Ovchinnikov DA, Wells CA, Wolvetang EJ. Concise Review: New Paradigms for Down Syndrome Research Using Induced Pluripotent Stem Cells: Tackling Complex Human Genetic Disease. Stem Cells Transl Med. 2013;2(3):175–84.

95. Lancaster MA, Knoblich JA. Organogenesis in a dish: Modeling development and disease using organoid technologies. Science (80-). 2014;345(6194).

96. Kelava I, Lancaster MA. Dishing out mini-brains: Current progress and future prospects in brain organoid research. Dev Biol. 2016;420(2):199–209.

97. Takebe T, Sekine K, Enomura M, Koike H, Kimura M, Ogaeri T, et al. Vascularized and functional human liver from an iPSC-derived organ bud transplant. Nature. 2013;499(7459):481–4.

98. Dye BR, Hill DR, Ferguson MA, Tsai YH, Nagy MS, Dyal R, et al. In vitro generation of human pluripotent stem cell derived lung organoids. Elife. 2015;2015(4):1–25.

99. Ilieva H, Polymenidou M, Cleveland DW. Non-cell autonomous toxicity in neurodegenerative disorders: ALS and beyond. J Cell Biol. 2009;187(6):761–72.

100. Kelava I, Lancaster MA. Stem Cell Models of Human Brain Development. Cell Stem Cell. 2016;18(6):736–48.

101. Lancaster MA. Brain organoids get vascularized. Nat Biotechnol. 2018;36(5):407–8.

102. Seiler A, Visan A, Buesen R, Genschow E, Spielmann H. Improvement of an in vitro stem cell assay for developmental toxicity: The use of molecular endpoints in the embryonic stem cell test. Reprod Toxicol. 2004;18(2):231–40.

103. Choi SM, Kim Y, Shim JS, Park JT, Wang R-H, Leach SD, et al. Efficient Drug Screening and Gene Correction for Treating Liver Disease. Hepatology. 2014;57(410):1–54.

104. Bahadduri PM, Polli JE, Swaan PW, Ekins S. Targeting Drug Transporters – Combining In Silico and In Vitro Approaches to Predict In Vivo. Vol. 637, Methods in Molecular Biology. 2010. 149–163 p.

105. Klein C, Nguyen D, Liu CH, Mizoguchi A, Bhan AK, Miki H, et al. Gene therapy for Wiskott-Aldrich syndrome: Rescue of T-cell signaling and amelioration of colitis upon transplantation of retrovirally transduced hematopoietic stem cells in mice. Blood. 2003;101(6):2159–66.

106. Wu SM, Hochedlinger K. Harnessing the potential of induced pluripotent stem cells for regenerative medicine. Nat Cell Biol. 2011;13(5):497–505.

107. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. Cell. 2007;131(5):861–72.

108. Kimbrel EA, Lanza R. Current status of pluripotent stem cells: moving the first therapies to the clinic. Nat Rev Drug Discov. 2015;14(10):681–92.

109. Scudellari M. A decade of : iPS Cells. Nature. 2016;534(7607):310–2.

110. Trounson A, DeWitt ND. Pluripotent stem cells progressing to the clinic. Nat Rev Mol Cell Biol. 2016;17(3):194–200.

111. Liu H, Kim Y, Sharkis S, Marchionni L, Jang YY. In vivo liver regeneration potential of human induced pluripotent stem cells from diverse origins. Sci Transl Med. 2011;3(82).

112. Lim WF, Inoue-Yokoo T, Tan KS, Lai MI, Sugiyama D. Hematopoietic cell differentiation from embryonic and induced pluripotent stem cells. Stem Cell Res Ther. 2013;4(3).

113. Okon EB, Karimi-abdolrezaee S, Hill CE, Sparling JS, Plemel JR, Plunet WT, et al. A Systematic Review of Cellular Transplantation Therapies for Spinal Cord Injury. J Neurotrauma. 2010;72:1–72.

114. Ross CA, Poirier MA. What is the role of protein aggregation in neurodegeneration? Nat Rev Mol Cell Biol. 2005;6(11):891–8.

115. Uryu K, Chen X, Martinez D, Browne KD, Johnson VE, Graham DI, et al. Multiple Proteins Implicated in Neurodegenerative Diseases Accumulate in Axons After Brain Trauma in Humans. Exp Neurol. 2007;208(2):185–92.

116. Silva MC, Haggarty SJ. Human pluripotent stem cell–derived models and drug screening in CNS precision medicine. Ann N Y Acad Sci. 2019;1–39.

117. Wan W, Cao L, Kalionis B, Xia S, Tai X. Applications of Induced Pluripotent Stem Cells in Studying the Neurodegenerative Diseases. Stem Cells Int. 2015;2015.

118. Ichida JK, Kiskinis E. Probing disorders of the nervous system using reprogramming approaches. EMBO J. 2015;34(11):1456–77.

119. Takazawa T, Croft GF, Amoroso MW, Studer L, Wichterle H, MacDermott AB. Maturation of spinal motor neurons derived from human embryonic stem cells. PLoS One. 2012;7(7):1–9.

120. Son EY, Ichida JK, Wainger BJ, Toma JS, Victor F, Woolf CJ, et al. Functional Integration of Dopaminergic Neurons Directly Converted from Mouse Fibroblasts. Cell Stem Cell. 2011;9(3):205–18.

121. Payne NL, Sylvain A, O'Brien C, Herszfeld D, Sun G, Bernard CCA. Application of human induced pluripotent stem cells for modeling and treating neurodegenerative diseases. N Biotechnol. 2015;32(1):212–28.

122. Israel MA, Yuan SH, Bardy C, Reyna SM, Mu Y, Herrera C, et al. Probing sporadic and familial Alzheimer's disease using induced pluripotent stem cells. Nature. 2012;482(7384):216–20.

123. Muratore CR, Rice HC, Srikanth P, Callahan DG, Shin T, Benjamin LNP, et al. The familial alzheimer's disease APPV717I mutation alters APP processing and Tau expression in iPSC-derived neurons. Hum Mol Genet. 2014;23(13):3523–36.

124. Sproul AA, Jacob S, Pre D, Kim SH, Nestor MW, Navarro-Sobrino M, et al. Characterization and molecular profiling of PSEN1 familial alzheimer's disease

iPSC-Derived neural progenitors. PLoS One. 2014;9(1).

125. Paquet D, Kwart D, Chen A, Sproul A, Jacob S, Teo S, et al. Efficient introduction of specific homozygous and heterozygous mutations using CRISPR/Cas9. Nature. 2016;533(7601):125–9.

126. Yagi T, Ito D, Okada Y, Akamatsu W, Nihei Y, Okano H, et al. Modeling familial alzheimer's disease with induced pluripotent stem cells. Clin Neurol. 2012;52(11):1134–6.

127. Mahairaki V, Ryu J, Peters A, Chang Q, Li T, Park TS, et al. Induced pluripotent stem cells from familial alzheimer's disease patients differentiate into mature neurons with amyloidogenic properties. Stem Cells Dev. 2014;23(24):2996–3010.

128. Liao MC, Muratore CR, Gierahn TM, Sullivan SE, Srikanth P, De Jager PL, et al. Single-cell detection of secreted Aβ and sAPPα from human IPSC-derived neurons and astrocytes. J Neurosci. 2016;36(5):1730–46.

129. Duan L, Bhattacharyya BJ, Belmadani A, Pan L, Miller RJ, Kessler JA. Stem cell derived basal forebrain cholinergic neurons from Alzheimer's disease patients are more susceptible to cell death. Mol Neurodegener. 2014;9(1):1–14.

130. Moore S, Evans LDB, Andersson T, Portelius E, Smith J, Dias TB, et al. APP Metabolism Regulates Tau Proteostasis in Human Cerebral Cortex Neurons. Cell Rep. 2015;11(5):689–96.

131. Raja WK, Mungenast AE, Lin YT, Ko T, Abdurrob F, Seo J, et al. Self-organizing 3D human neural tissue derived from induced pluripotent stem cells recapitulate Alzheimer's disease phenotypes. PLoS One. 2016;11(9):1–18.

132. Choi SH, Kim YH, Hebisch M, Sliwinski C, Lee S, Avanzo CD, et al. A three-dimensional human neural cell culture model of Alzheimer's disease. Nature. 2014;515(7526):274–8.

133. Kim YH, Choi SH, D'Avanzo C, Hebisch M, Sliwinski C, Bylykbashi E, et al. A 3D human neural cell culture system for modeling Alzheimer's disease. Nat Protoc. 2015;10(7):985–1006.

134. Espuny-Camacho I, Arranz AM, Fiers M, Snellinx A, Ando K, Munck S, et al. Hallmarks of Alzheimer's Disease in Stem-Cell-Derived Human Neurons Transplanted into Mouse Brain. Neuron. 2017;93(5):1066–81.

135. Balez R, Steiner N, Engel M, Muñoz SS, Lum JS, Wu Y, et al. Neuroprotective

effects of apigenin against inflammation, neuronal excitability and apoptosis in an induced pluripotent stem cell model of Alzheimer's disease. Sci Rep. 2016;6(August):1–16.

136. Brownjohn PW, Smith J, Portelius E, Serneels L, Kvartsberg H, De Strooper B, et al. Phenotypic Screening Identifies Modulators of Amyloid Precursor Protein Processing in Human Stem Cell Models of Alzheimer's Disease. Stem Cell Reports. 2017;8(4):870–82.

137. Kimura J, Shimizu K, Kajima K, Yokosuka A, Mimaki Y, Oku N, et al. Nobiletin reduces intracellular and extracellular β-amyloid in iPS cell-derived Alzheimer's disease model neurons. Biol Pharm Bull. 2018;41(4):451–7.

138. Bright J, Hussain S, Dang V, Wright S, Cooper B, Byun T, et al. Human secreted tau increases amyloid-beta production. Neurobiol Aging. 2015;36(2):693–709.

139. Takamatsu K, Ikeda T, Haruta M, Matsumura K, Ogi Y, Nakagata N, et al. Degradation of amyloid beta by human induced pluripotent stem cell-derived macrophages expressing Neprilysin-2. Stem Cell Res. 2014;13(3):442–53.

140. Fujiwara N, Shimizu J, Takai K, Arimitsu N, Saito A, Kono T, et al. Restoration of spatial memory dysfunction of human APP transgenic mice by transplantation of neuronal precursors derived from human iPS cells. Neurosci Lett. 2013;557(PB):129–34.

141. Tong LM, Djukic B, Arnold C, Gillespie AK, Yoon SY, Wang MM, et al. Inhibitory interneuron progenitor transplantation restores normal learning and memory in ApoE4 knock-in mice without or with Aβ accumulation. J Neurosci. 2014;34(29):9506–15.

142. Brown RH, D.Phil. MD, Al-Chalabi A. Amyotrophic Lateral Sclerosis. N Engl J Med. 2017;377:162–72.

143. Dimos JT, Rodolfa KT, Niakan KK, Weisenthal LM, Mitsumoto H, Chung W, et al. Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. Science (80-). 2008;321(5893):1218–21.

144. Mitne-Neto M, Machado-Costa M, Marchetto MCN, Bengtson MH, Joazeiro CA, Tsuda H, et al. Downregulation of VAPB expression in motor neurons derived from induced pluripotent stem cells of ALS8 patients. Hum Mol Genet. 2011;20(18):3642–52.

145. Egawa N, Kitaoka S, Tsukita K, Naitoh M, Takahashi K, Yamamoto T, et al.

Drug screening for ALS using patient-specific induced pluripotent stem cells. Sci Transl Med. 2012;4(145).

146. Popescu IR, Nicaise C, Liu S, Bisch G, Knippenberg S, Daubie V, et al. Neural Progenitors Derived From Human Induced Pluripotent Stem Cells Survive and Differentiate Upon Transplantation Into a Rat Model of Amyotrophic Lateral Sclerosis. Stem Cells Transl Med. 2013;2(3):167–74.

147. Higelin J, Demestre M, Putz S, Delling JP, Jacob C, Lutz AK, et al. FUS mislocalization and vulnerability to DNA damage in ALS patients derived hiPSCs and aging motoneurons. Front Cell Neurosci. 2016;10(DEC2016):1–21.

148. Kiskinis E, Sandoe J, Williams LA, Boulting GL, Moccia R, Wainger BJ, et al. Pathways disrupted in human ALS motor neurons identified through genetic correction of mutant SOD1. Cell Stem Cell. 2014;14(6):781–95.

149. Wainger BJ, Kiskinis E, Mellin C, Wiskow O, Han SSW, Sandoe J, et al. Intrinsic membrane hyperexcitability of amyotrophic lateral sclerosis patient-derived motor neurons. Cell Rep. 2014;7(1):1–11.

150. Burkhardt MF, Martinez FJ, Wright S, Ramos C, Volfson D, Mason M, et al. A cellular model for sporadic ALS using patient-derived induced pluripotent stem cells. Mol Cell Neurosci. 2013;33(8):839–41.

151. Devlin AC, Burr K, Borooah S, Foster JD, Cleary EM, Geti I, et al. Human iPSC-derived motoneurons harbouring TARDBP or C9ORF72 ALS mutations are dysfunctional despite maintaining viability. Nat Commun. 2015;6:1–12.

152. Janos TO. Ezogabine treatment shown to reduce motor neuron excitability in ALS patients. Massachusetts General Hospital - News Release. Massachusetts; 2018. p. 1–2.

153. Nizzardo M, Simone C, Rizzo F, Ruggieri M, Salani S, Riboldi G, et al. Minimally invasive transplantation of iPSC-derived ALDHhiSSCloVLA41 neural stem cells effectively improves the phenotype of an amyotrophic lateral sclerosis model. Hum Mol Genet. 2014;23(2):342–54.

154. Lees AJ, Hardy J, Revesz T. Parkinson’s disease. Lancet. 2009;(373):2055–66.

155. Mishima T, Fujioka S, Fukae J, Yuasa-Kawada J, Tsuboi Y. Modeling Parkinson’s Disease and Atypical Parkinsonian Syndromes Using Induced Pluripotent Stem Cells. Int J Mol Sci. 2018;19(12):3870.

156. Nguyen HN, Byers B, Cord B, Shcheglovitov A, Byrne J, Gujar P, et al. LRRK2

Mutant iPSC-Derived DA Neurons Demonstrate Increased Susceptibility to Oxidative Stress. Cell Stem Cell. 2011;8(3):267–280.

157. Yi F, Xu X, Ruiz S, Zhang W, Ren B, Wagner U, et al. Progressive degeneration of human neural stem cells caused by pathogenic LRRK2. Nature. 2013;491(7425):603–7.

158. Sánchez-Danés A, Richaud-Patin Y, Carballo-Carbajal I, Jiménez-Delgado S, Caig C, Mora S, et al. Disease-specific phenotypes in dopamine neurons from human iPS-based models of genetic and sporadic Parkinson's disease. EMBO Mol Med. 2012;4(5):380–95.

159. Cooper O, Seo H, Andrabi S, Guardia-Laguarta C, Graziotto J, Sundberg M, et al. Pharmacological rescue of mitochondrial deficits in iPSC-derived neural cells from patients with familial Parkinson's disease. Sci Transl Med. 2012;4(141).

160. Devine MJ, Ryten M, Vodicka P, Thomson AJ, Burdon T, Houlden H, et al. Parkinson's disease induced pluripotent stem cells with triplication of the α-synuclein locus. Nat Commun. 2011;2(1):410–40.

161. Kouroupi G, Taoufik E, Vlachos IS, Tsioras K, Antoniou N, Papastefanaki F, et al. Defective synaptic connectivity and axonal neuropathology in a human iPSC-based model of familial Parkinson's disease. Proc Natl Acad Sci. 2017;114(18):3679–88.

162. Klein C, Westenberger A. Genetics of Parkinson's Disease. Cold Spring Harb Perspect Med. 2012;2:1–16.

163. Woodard CM, Campos BA, Kuo S, Nirenberg MJ, Nestor MW, Zimmer M, et al. iPS Cell-Derived Dopamine Neurons Reveal Differences between Monozygotic Twins Discordant for Parkinson's Disease. Cell Rep. 2014;9(4):1173–82.

164. Soldner F, Stelzer Y, Shivalila CS, Abraham BJ, Latourelle JC, Inmaculada Barrasa M, et al. Parkinson-associated risk variant in enhancer element produces subtle effect on target gene expression HHS Public Access. Nature. 2016;533(7601):95–9.

165. Chung CY, Khurana V, Auluck PK, Tardiff DF, Mazzulli JR, Soldner F, et al. Identification and Rescue of a-Synuclein Toxicity in Parkinson Patient–Derived Neurons. Science (80-). 2013;342(6161):983–7.

166. Burbulla LF, Song P, Mazzulli JR, Zampese E, Wong YC, Jeon S, et al. Dopamine oxidation mediates mitochondrial and lysosomal dysfunction in

Parkinson’s disease. Science (80-). 2017;357(6357):1255–61.

167. Mittal S, Bjørnevik K, Im DS, Flierl A, Dong X, Locascio JJ, et al. β2-Adrenoreceptor is a regulator of the α-synuclein gene driving risk of Parkinson’s disease. Science (80-). 2017;357(6354):891–8.

168. Sommer A, Maxreiter F, Krach F, Fadler T, Grosch J, Maroni M, et al. Th17 Lymphocytes Induce Neuronal Cell Death in a Human iPSC-Based Model of Parkinson’s Disease. Cell Stem Cell. 2018;23(1):123-131.e6.

169. Takahashi J. Strategies for bringing stem cell-derived dopamine neurons to the clinic: The Kyoto trial. 1st ed. Vol. 230, Progress in Brain Research. Elsevier B.V.; 2017. 213–226 p.

170. Hallett PJ, Deleidi M, Astradsson A, Smith GA, OliverCooper, Osborn T, et al. Successful function of autologous iPSC-derived dopamine neurons following transplantation in a non-human primate model of Parkinson’s disease. Cell Stem Cell. 2015;16(3):269–274.

171. Kikuchi T, Morizane A, Doi D, Magotani H, Onoe H, Hayashi T, et al. Human iPS cell-derived dopaminergic neurons function in a primate Parkinson’s disease model. Nature. 2017;548(7669):592–6.

172. Takahashi J, Price-Evans A. Preparing for first human trial of induced pluripotent stem cell-derived cells for Parkinson’s disease: An interview with Jun Takahashi. Regenerative Medicine. 2019;

173. UMIN Kyoto Trial to Evaluate the Safety and Efficacy of iPSC-Derived Dopaminergic Progenitors in the Treatment of Parkinson’s Disease [Internet]. [cited 2019 Sep 13]. Available from: https://upload.umin.ac.jp/cgi-open-bin/ctr_e/ctr_view.cgi?recptno=R000038278

174. Kim K, Doi A, Wen B, Ng K, Zhao R, Cahan P, et al. Epigenetic memory in induced pluripotent stem cells. Nature. 2010;467(7313):285–90.

175. Rouhani F, Kumasaka N, de Brito MC, Bradley A, Vallier L, Gaffney D. Genetic Background Drives Transcriptional Variation in Human Induced Pluripotent Stem Cells. PLoS Genet. 2014;10(6).

176. Zhang G, Shang B, Yang P, Cao Z, Pan Y, Zhou Q. Induced pluripotent stem cell consensus genes: Implication for the risk of tumorigenesis and cancers in induced pluripotent stem cell therapy. Stem Cells Dev. 2012;21(6):955–64.